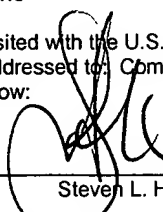


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March 9, 2004  
Date

  
Steven L. Highlander

**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

Gary L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHODS AND COMPOSITIONS FOR  
THE DIAGNOSIS AND TREATMENT OF  
CANCER

Group Art Unit: 1632

Examiner: Joseph T. Woitach

Atty. Dkt. No.: INRP:041/SLH

**APPEAL BRIEF**

**BOX AF**

Commissioner for Patents  
PO Box 1450  
Alexandria, VA 22313-1450

Commissioner:

Appellant hereby submits an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the Office Action dated December 9, 2003. The fee for filing this Appeal Brief is \$330, and is attached hereto.

If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed material, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/INRP:041US/SLH.

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## **I. REAL PARTY IN INTEREST**

The real parties in interest are the assignee, The Board of Regents, University of Texas System, the exclusive licensee, Introgen Therapeutics.

## **II. RELATED APPEALS AND INTERFERENCES**

There are no related appeals or interferences.

## **III. STATUS OF THE CLAIMS**

Claims 1-25 were filed with the original application. Claims 26-150 have been added. Claims 15, 21-25, 78, and 79 were canceled in the first response; claims 33-35, 69-72, 104-107, 133-136, 140, 141, 144, and 145 were canceled in an amendment filed concurrent with the first Appeal Brief; and claims 38-68, 73-77, 80-103, 108-132, 137-139, 142, and 143 were canceled in an amendment filed concurrent with the Reply Brief. Claim 10 was canceled in the Preliminary Amendment on Remand. Thus, claims 1-9, 11-14, 16-20, 26-32, 36, 37, and 146-150 remain pending and are appealed. A copy of the pending claims is attached as Appendix 1.

## **IV. STATUS OF AMENDMENTS**

No amendments are pending and unentered.

## **V. SUMMARY OF THE INVENTION**

The present invention deals with cancer gene therapy. More particularly, it addresses the use of adenoviral-p53 vectors for the treatment of various forms of cancer. Specification at page 3, lines 12-21. In a particular embodiment, the invention involves the use of a continuous perfusion protocol to treat a tumor site over a period of time. Specification at page 4, lines 8-11. In a second embodiment, the invention provides for treatment of microscopic residual disease, resulting from tumor resection. Specification at page 4, lines 2-4. In various dependent

embodiments, the invention relates to treatment of both p53-mutated and p53-wild-type tumors. Specification at page 3, line 24.

## **VI. ISSUES ON APPEAL**

A. Does the subject matter of claims 1-14, 16-20, 26-32, 36, and 37 constitute double patenting over co-pending Application No. 09/968,958?

B. Are claims 1-9, 13, 14, 16-20, and 36 anticipated by Liu *et al.* (1994) (Exhibit A)?

C. Are claims 1-9, 13, 14, 16-20, and 36 anticipated by Clayman *et al.* (1995) (Exhibit B)?

D. Are claims 1-14, 16-20, 26-32, 36, and 37 anticipated by either U.S. Patent No. 5,747,469 (Exhibit C) or U.S. Patent No. 6,017,524 (Exhibit D)?

E. Are claims 1-14, 16-20, 26-32, 36, and 37 obvious over Clayman *et al.* and Liu *et al.* in view of Zhang *et al.* (Exhibit E)?

F. Are claims 1-14, 16-20, 26-32, 36, and 37 obvious over Srivastava *et al.* (Exhibit F), Cajot *et al.* (Exhibit G), Katayose *et al.* (Exhibit H), Wills *et al.* (Exhibit I), Liu *et al.*, and Zhang *et al.*?

## **VII. GROUPING OF THE CLAIMS**

The claims do not stand and fall together. With respect to the rejection of claims 1-9, 13, 14, 16-20, and 36 under 35 U.S.C. § 102(b) over Liu *et al.*, Appellant submits that claims 4, 13, 17, 18, 19-20, and 36 are separately patentable, and they have been dealt with separately in Section IX.E.1. With respect to the rejection of claims 1-9, 13, 14, 16-20, and 36 under 35 U.S.C. § 102(b) over Clayman *et al.*, Appellant submits that claims 4, 13, 17, 18, 19-20, and 36

are separately patentable, and they have been dealt with separately in Section IX.E.2. With respect to the rejection of claims 1-14, 16-20, 26-32, 36, and 37 under 35 U.S.C. § 102(b) over Roth *et al.*, Appellant submits that claims 12, 19 and 20 are separately patentable, and they have been dealt with separately in Section IX.E.3. With respect to the rejection of claims 1-14, 16-20, 26-32, 36, and 37 under 35 U.S.C. § 103(a) over Clayman *et al.* and Liu *et al.*, in view of Zhang *et al.*, Appellant submits that claims 4, 11 and 26, 12, 13, 17, 18, 19-20, 27, 29-30, 36, and 37 are separately patentable, and they have been dealt with separately in Section IX.E.4. With respect to the rejection of claims 1-14, 16-20, 26-32, 36, and 37 under 35 U.S.C. § 103(a) over Srivastava *et al.*, Cajot *et al.*, Katayose *et al.*, Wills *et al.*, Liu *et al.*, and Zhang *et al.*, Appellant submits that claims 4, 12, 13, 17, 18, 19-20, 27, 29-30, 36, and 37 are separately patentable, and they have been dealt with separately in Section IX.E.5.

### VIII. SUMMARY OF THE ARGUMENT

1. The Examiner made a provisional, non-statutory double patenting rejection of claims 1-14, 16-20, 26-32, 36, and 37 over claims 26-88 of co-pending Application No. 09/968,958. Appellant will submit a terminal disclaimer, if appropriate, when allowable subject matter is determined.

2. In the first anticipation rejection, the Examiner rejected claims 1-9, 13, 14, 16-20, and 36 over Liu *et al.* It is Appellant's position that a *prima facie* case has not been made because the Examiner failed to show that each and every element of the claimed invention is described in the prior art reference. At a minimum, the Examiner failed to show where Liu teaches (1) "a tumor cell expressing wild-type p53" or (2) the treatment of a "human subject."

*Tumor cell expressing wild-type p53.* Liu appears to teach only the use of the Ad-p53 construct to compensate for the absence of a functional p53 molecule in tumors lacking that molecule. The Examiner failed to show where Liu teaches the treatment of a tumor cell having a functional p53 molecule using an expression construct that, itself, encodes a p53 molecule.

*Treatment of a human subject.* The Examiner failed to show where Liu teaches a method for the treatment of a human subject.

3. In the second anticipation rejection, the Examiner rejected claims 1-9, 13, 14, 16-20, and 36 over Clayman *et al.* This reference was published less than one year before the November 30, 1995 priority date of the present application. Appellant has attached a copy of Dr. Clayman's previously submitted declaration (Exhibit J) evidencing that Clayman *et al.* was not "by another," and therefore not prior art under § 102(a).

Even if the Clayman reference was available as prior art, a *prima facie* case of anticipation has not been made because the Examiner failed to show that each and every element of the claimed invention is taught by the reference. For example, the Examiner failed to show where Clayman teaches a method for treating a tumor cell expressing wild-type p53 in a "human subject."

4. In the third anticipation rejection, the Examiner rejected claims 1-14, 16-20, 26-32, 36, and 37 over Roth *et al.* (either U.S. Patent 5,747,469 or 6,017,524). A *prima facie* case has not been made because the Examiner failed to show where Roth inhibited the growth of a tumor cell expressing wild-type p53 in a human subject.

5. In the first obviousness rejection, the Examiner rejected claims 1-14, 16-20, 26-32, 36, and 37 over Clayman *et al.* and Liu *et al.* in view of Zhang *et al.* It is Appellant's position that a *prima facie* case of obviousness has not been made because the Examiner failed to show that every element of the claimed invention is described in the prior art references. Clayman is not available as prior art, and the Examiner failed to show where Liu and Zhang teach the treatment of a tumor cell expressing wild-type p53 in a human subject.

*Clayman is not available as prior art.* Clayman was published less than one year before the November 30, 1995 priority date of the present application. Appellant has attached a copy of Dr. Clayman's previously submitted declaration evidencing that Clayman *et al.* was not "by another," and therefore not prior art under § 102(a).

*Tumor cells expressing wild-type p53.* The Examiner failed to show that combining the teachings of Liu with the teachings of Zhang teaches a method of inhibiting growth of a tumor cell that itself expresses wild-type p53. Liu appears to teach only inhibiting the growth of a tumor cell lacking a functional p53 molecule. Similarly, Zhang appears to teach only the replacement of p53 in cells lacking p53 or expressing a mutant p53.

6. In the second obviousness rejection, the Examiner rejected claims 1-14, 16-20, 26-32, 36, and 37 over Srivastava *et al.*, Cajot *et al.*, Katayose *et al.*, Wills *et al.*, Liu *et al.*, and Zhang *et al.* It is Appellant's position that a *prima facie* case of obviousness has not been made.

*Srivastava is not prior art.* Srivastava is not available as prior art because it was published after the priority date of the present invention.

*In light of Dr. Clayman's Rule 131 declaration, Srivastava and Katayose are not properly citable against the present invention. Dr. Clayman's Rule 131 declaration (Exhibit K) avers conception of the present invention prior to August 11, 1995, the date of the earliest publication as between Srivastava and Katayose, and diligence to Appellant's filing date in November, 1995.*

*Srivastava and Katayose teach away from the treatment of p53-positive tumor cells with vectors expressing p53. Srivastava and Katayose evidence confusion in the field with regard to the treatment of p53-positive tumor cells with vectors expressing p53 and, in fact, teach away from such an approach.*

*Cajot is scientifically flawed. Cajot is scientifically flawed such that those skilled in the art would not rely on its teachings.*

*There is no reasonable basis for extrapolating from in vitro and animal studies to human clinical therapy. Because of the differences between in vitro and in vivo studies, and the differences between in vivo animal models and human clinical applications, especially when simultaneously making the leap from treating p53-negative to p53-positive cells, those of skill in the art would not have had a reasonable expectation of success based on the prior art.*

## **IX. ARGUMENT**

### **A. Substantial Evidence is Required to Uphold the Examiner's Position.**

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by

“substantial evidence” within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that “the ‘substantial evidence’ standard asks whether a reasonable fact finder could have arrived at the agency’s decision.” *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner’s position on Appeal must be supported by “substantial evidence” within the record in order to be upheld by the Board of Patent Appeals and Interferences.

**B. The Provisional Double Patenting Rejection**

Claims 1-14, 16-20, 26-32, 36, and 37 are provisionally rejected for non-statutory double patenting over claims 26-88 of co-pending Application No. 09/968,958. Appellant will submit a terminal disclaimer, if appropriate, when allowable subject matter is determined.

**C. Rejections Under 35 U.S.C. § 102**

**1. The Legal Standard for Anticipation.**

Anticipation requires that each and every element of the claimed invention be described, either expressly or inherently, in a single prior art reference. *Telemac Cellular Corp. v. Topp Telecom, Inc.*, 247 F.3d 1316, 1327, 58 U.S.P.Q.2d 1545, 1552 (Fed. Cir. 2001); *Verdegaal Bros., Inc. v. Union Oil Co.*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987). It is well settled that the burden of establishing a *prima facie* case of anticipation resides with the Examiner and only if that burden is met, does the burden of going forward shift to the Appellant. See *In re Sun*, 31 U.S.P.Q.2d 1451 (Fed. Cir. 1993). Appellant submits that the Examiner has not established a *prima facie* case.



## 2. The Liu *et al.* Reference

Claims 1-9, 13, 14, 16-20, and 36 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Liu *et al.* Liu is said to teach a method of reducing tumor burden in a mouse following the administration of an adenoviral vector encoding a wild-type p53 polypeptide. Appellant traverses.

### a) *Liu Does Not Teach Every Element of the Claimed Invention*

The presently claimed invention is directed to a method of inhibiting growth of a tumor cell “*expressing wild-type p53*” in a “*human subject*.” The Examiner has failed to show that Liu teaches these elements.

The experiments described in Liu use the SCCHN cell lines Tu-138 and Tu-177, both of which possess a *mutated* p53 gene (see p. 3663, col. 2). In other words, Liu uses the Ad-p53 construct to compensate for the absence of a functional p53 molecule in tumors lacking that molecule. The Examiner has failed to show where Liu teaches that a tumor cell having a functional p53 molecule may be treated using an expression construct that, itself, encodes a p53 molecule. Furthermore, the Examiner has failed to show where Liu teaches the treatment of a *human subject*. Liu appears to teach the treatment of tumor cells only *in vitro* or in a nude mouse model. Thus, a *prima facie* case of anticipation has not been made because the Examiner has not shown that the reference teaches every element of the claims.

In addition, for the reasons stated in section IX.E.1 below, Appellant submits that claims 4, 13, 17, 18, 19 and 20, and 36 are separately patentable.

**b) Conclusion**

The Examiner has failed to show, at a minimum, that Liu teaches "a tumor cell expressing wild-type p53" or the treatment of a "human subject." Thus, it is submitted that the rejection of claims 1-9, 13, 14, 16-20, and 36 is fatally defective in failing to provide a disclosure of every element of the claimed invention. Appellant requests that the Board reverse this rejection.

**3. The Clayman *et al.* Reference**

Claims 1-9, 13, 14, 16-20, and 36 stand rejected under 35 U.S.C. § 102(b) as anticipated by Clayman *et al.* The Examiner does not specify which Clayman *et al.* reference is being relied upon, but based on the Examiner's description of the reference's teachings it appears to be the 1995 publication. This reference was published less than one year before the November 30, 1995 priority date of the present application. Accordingly, this rejection should be brought under § 102(a).

Appellant notes that a declaration (Exhibit J) was submitted with the response filed August 17, 1998 evidencing that Clayman *et al.* was not "by another," and therefore not prior art under § 102(a). Appellant, therefore, requests that the Board reverse this rejection.

Even if the Clayman reference was available as prior art, a *prima facie* case of anticipation has not been made because the Examiner failed to show that each and every element of the claimed invention is taught by the reference. The presently claimed invention is directed to a method of inhibiting growth of a tumor cell expressing wild-type p53 in a "*human subject*." The Examiner failed to show where Clayman teaches a method for treating a tumor cell expressing wild-type p53 in a "human subject."

In addition, for the reasons stated in section IX.E.2 below, Appellant submits that claims 4, 13, 17, 18, 19 and 20, and 36 are separately patentable.

**4. U.S. Patents 5,747,469 and 6,017,524**

Claims 1-14, 16-20, 26-32, 36, and 37 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Roth *et al.* (either U.S. Patent 5,747,469 or 6,017,524). Appellant notes that although this rejection was advanced under § 102(b), the Examiner probably intended to advance the rejection under § 102(e). Roth is said to teach a method of treating a tumor by killing the cells of the tumor through the expression of p53. Roth is also said to teach that p53 can be delivered by a variety of vectors, including adenoviral vectors, and in conjunction with known chemotherapeutic agents and protocols. Appellant traverses this rejection.

**a) *The Roth Patents Do Not Teach All of the Elements of the Claimed Invention***

As stated above, the presently claimed invention is directed to a method of inhibiting growth of a tumor cell expressing wild-type p53 in a human subject. The Examiner has failed to show where Roth inhibited the growth of a tumor cell expressing wild-type p53 in a human subject. Thus, a *prima facie* case for anticipation has not been made.

In addition, for the reasons stated in section IX.E.3 below, Appellant submits that claims 12, 19 and 20 are separately patentable.

**b) *Conclusion***

The Examiner has failed to show where Roth inhibited the growth of a tumor cell expressing wild-type p53 in a human subject. Thus, it is submitted that the rejection of claims 1-

14, 16-20, 26-32, 36, and 37 is fatally defective in failing to provide a disclosure of every element of the claimed invention. Appellant requests that the Board reverse this rejection.

#### **D. Rejections Under 35 U.S.C. § 103**

##### **1. The Legal Standard for Obviousness**

Appellant respectfully notes the high standard by which an obviousness argument based on a combination of references is judged. "All words in a claim must be considered in judging the patentability of that claim against the prior art." *In re Wilson*, 424 F.2d 1382, 1385 (C.C.P.A. 1970). And, *all* of the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 985 (C.C.P.A. 1974). In addition, in the case of *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991), the Federal Circuit stated that an Examiner must establish two criteria in order to make a *prima facie* case of obviousness:

- 1) the prior art would have suggested to one of ordinary skill in the art to make the composition as claimed; and
- 2) the prior art demonstrates a reasonable expectation of success of the invention.

*Vaeck* also emphasized that both the suggestion and reasonable expectation of success must be found in the prior art, not in the Appellant's disclosure.

When an obviousness determination is based on multiple prior art references, there must be a showing of some "teaching, suggestion, or reason" to combine the references. *Gambro Lundia AB v. Baxter Healthcare Corp.*, 110 F.3d 1573, 1579 (Fed. Cir. 1997) (also noting that the "absence of such a suggestion to combine is dispositive in an obviousness determination").

Furthermore, the burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious

in light of the prior art. *See Manual of Patent Examining Procedure* § 2144.03; *Graham v. John Deere Co.*, 383 U.S. 1, 18 (1966). If the Examiner fails to establish a *prima facie* case of obviousness, the rejection is improper and will be overturned. *In re Rijckaert*, 9 F.3d 1531, 1532 (Fed. Cir. 1993). Even if the Examiner properly meets the burden of showing a *prima facie* case of obviousness, the Appellant may still overcome the obviousness rejection through a showing of secondary considerations such as unexpected results, long felt need, failure by others or commercial success. *See Graham* at 17-18.

**2. The Claimed Invention is Not Obvious in View of the Clayman *et al.*, Liu *et al.*, and Zhang *et al.* References**

Claims 1-14, 16-20, 26-32, 36, and 37 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Clayman *et al.* and Liu *et al.* in view of Zhang *et al.* The Examiner argues that both Liu and Clayman teach a method of reducing tumor burden in a mouse following the administration of an adenoviral vector encoding a wild-type p53 polypeptide, and that the vector may be delivered surgically to a revealed tumor in 100  $\mu$ l volumes in increasing log increments up to  $10^{12}$  PFU. The Examiner states that Clayman and Liu do not teach the specific volumes or spacing for the injections nor do they teach the treatment of cancer using p53 therapy in combination with other methods of cancer treatment. The Examiner argues, however, that multiple injections and various volumes would have been obvious to those of skill in the art based on the size and shape of the tumor, and that the combination of p53 therapy with other cancer treatments would have been obvious in view of Zhang *et al.* Appellant traverses this rejection.

a) *The Clayman et al. Reference is Not Prior Art*

As noted above, the declaration from Dr. Gary Clayman evidences that the non-inventor co-authors of the Clayman *et al.* reference acted under the direction of Dr. Clayman, provided starting materials, or rendered editorial assistance to Dr. Clayman. Thus, Clayman *et al.* cannot properly be considered “by another” and therefore it cannot be the basis of a rejection under § 103(a).

b) *Liu and Zhang Do Not Teach All of the Elements of the Claimed Invention*

To establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 985 (C.C.P.A. 1973). The claimed invention is directed to a method of inhibiting growth of a tumor cell expressing *wild-type* p53. The Examiner has failed to show where the prior art references teach the treatment of tumor cells expressing wild-type p53.

As described above, the Examiner has failed to show that Liu teaches a method of inhibiting the growth of a tumor cell expressing *wild-type* p53. Combining the teachings of Liu with the teachings of Zhang still fails to teach a method of inhibiting growth of a tumor cell that itself expresses *wild-type* p53. The Zhang reference is a review article on gene therapy for cancer. Zhang appears to teach only the replacement of p53 in cells lacking p53 or expressing a mutant p53; it does not appear to teach p53 supplementation in cells expressing wild-type p53. With regard to p53 gene therapy, Zhang states, “research in this area is progressing rapidly and has demonstrated that the *restoration* of wild-type p53 function in tumour cells could be a very efficient approach to cancer therapy.” Zhang *et al.*, p. 494, col. 2 (emphasis added). This statement reinforces that the conventional thinking at the time was that Ad-p53 gene therapy

would only be beneficial in tumors lacking a functional p53 molecule. There does not appear to be any suggestion that a tumor cell having a functional p53 molecule may be treated using an expression construct that, itself, encodes a p53 molecule. Thus, the Examiner has failed to show that Liu and Zhang teach or suggest the treatment of tumor cells that express wild-type p53.

In addition, for the reasons stated in section IX.E.4 below, Appellant submits that claims 4, 11 and 26, 12, 13, 17, 18, 19 and 20, 26, 27, 29 and 30, 36, and 37 are separately patentable.

**c) Conclusion**

The Examiner has not made a *prima facie* case that the claimed invention is obvious in view of Clayman, Liu, and Zhang. First, Clayman is not prior art, and thus an obviousness rejection based on this reference is improper. Second, the Examiner has failed to show that Liu and Zhang teach or suggest the treatment of p53-positive tumor cells. Thus, the references do not combine to teach all of the elements of the invention. Appellant, therefore, requests that the Board reverse this rejection.

**3. The Claimed Invention is Not Obvious in View of the Srivastava *et al.*, Cajot *et al.*, Katayose *et al.*, Wills *et al.*, Liu *et al.*, and Zhang *et al.* References**

Claims 1-14, 16-20, 26-32, 36, and 37 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Srivastava *et al.*, Cajot *et al.*, Katayose *et al.*, Wills *et al.*, Liu *et al.*, and Zhang *et al.* The Examiner asserts that Srivastava teaches a method of inhibiting the growth of wild-type p53 expressing LNCaP cells with adenoviral vectors encoding and expressing p53, and that treatment with such vectors can be practiced *in vivo*. It is then asserted that Cajot and Katayose provide *in vitro* evidence that analogous methods work in other cell types, namely Hut292DM and MCF-1. Wills is said to teach the repetitive delivery of adenoviral vectors encoding wild-

type p53 for the inhibition of tumor growth in an animal, and that it teaches the combination of the treatment with p53 expressing vectors with conventional cancer treatments. Zhang is said to specifically teach that gene therapy can be complemented by conventional methods of cancer treatment. Appellant traverses this rejection.

**a) *Srivastava is Not Available as Prior Art***

Appellant notes that Srivastava was published in December, 1995, and therefore is not even available as prior art to the present case under 35 U.S.C. § 102(a). Even if it were available as prior art, Srivastava would still not be relevant for the various reasons that have been previously set forth and enumerated herein.

**b) *The Rule 131 Declaration Shows That Dr. Clayman Had Obtained an IND and Initiated the Human Clinical Trial Process Before the Publication of Katayose or Srivastava***

Appellant filed a new declaration under Rule 131 on June 5, 2002 (Exhibit K) directed towards documentation that the inventor was involved in setting up and performing clinical trials in human patients directed at the study of the effects of Ad-WTp53 gene therapy on both wild-type and mutated p53 tumor cells during critical time periods. This declaration avers conception of the present invention prior to August 11, 1995, the date of the earliest publication as between Katayose and Srivastava, and diligence to Appellant's filing date in November, 1995. Within the declaration, the following documents dated prior to August 11, 1995 were cited in support of conception:<sup>1</sup>

1. A transcribed tape of a Grand Rounds Seminar citing the success in treating both wild-type and mutated p53 in tumor cells in animals (paragraph 5A of the



Clayman Declaration, Exhibit 1) and indicating that his focus is on the development of novel molecular therapies for treating cancer. Please note that **bolded** portions of this presentation which indicate his findings that the p53 therapy worked to slow or inhibit tumor growth in both cellular and in animal models of human tumors. It should be noted that the date of this Grand Rounds presentation to a group of clinicians at MD Anderson was more than one year prior to the November, 1995 filing date. It included the showing of slides, but no handouts were given out and there was no abstract of this presentation prepared or published.

2. There is a document dated prior to August 11, 1995 demonstrating approval from the Food and Drug Administration ("FDA") and the Institutional Review Board of MD Anderson Cancer Center ("IRB") for the use of adenovirus treatment and for the initial protocol submission (paragraph 5C, Exhibit 2).
3. The final version of the approved informed consent form (paragraph 5D, Exhibit 3)
4. The final version of the protocol used in the clinical study indicating Ad-WTp53 induced apoptosis in tumor cells regardless of p53 status is attached as Exhibit 4 to the Clayman declaration, and discussed in paragraphs 5E and 5F of the declaration. In this protocol, Dr. Clayman observes that his laboratory studies had shown that head and neck squamous cell carcinomas ("HNSCC") underwent apoptosis (cell death) when treated with Ad-WTp53, regardless of endogenous

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<sup>1</sup> The attachments have all been redacted to remove certain names and also to remove all dates prior to August 11, 1995. All of the date redactions have been designated with an "\*".

p53 status, and indicates that “[t]hese results support the use of this strategy in a clinical trial.” See Protocol, page 3, second full paragraph. For this reason, the study was designed to include patients having HNSCC regardless of p53 endogenous status of the tumor, and to assess the tumor for its p53 status. See, e.g., Protocol, page 11, section 6.7.

5. Additional documents were cited to prove diligence after conception: Approval from the FDA for the Investigational New Drug (“IND”) of Ad-WTp53 (paragraph 5G, Exhibit 5); various documents inquiring and granting approval for revised versions of the protocol used in the clinical study (paragraphs 5H-J, Exhibits 5-8); documentation showing IRB approval for the clinical study (paragraph 5K, Exhibit 9); and treatment of patients ( paragraph 5L). Also, this declaration highlights the irrelevancy of the Katayose and Srivastava references due to their lack of experiments involving human subjects, their inconsistencies within their own data sets, and the fact that they teach away from examining wild-type p53 tumors. In light of this declaration, Appellant again submits that Katayose and Srivastava are not properly citable against the present invention.

*c)      Srivastava and Katayose Teach Away*

Even if Srivastava and Katayose were available as prior art, they still would not support the obviousness rejection. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on the Appellant’s disclosure. *In re Vaeck*, 947 F.2d 488, 493 (Fed Cir. 1991). A prior art reference that “teaches away” from the claimed invention is a significant factor to be considered in determining obviousness. *In re Gurley*, 27 F.3d 551, 554 (Fed. Cir. 1994). A “reference may be

said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Appellant.” *Tec Air Inc. v. Denso Mfg. Michigan Inc.*, 192 F.3d 1353, 1360 (Fed. Cir. 1999) (citing with approval, *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994)). Srivastava and Katayose teach away from the treatment of p53-positive cells with a p53 expression construct.

Srivastava demonstrates the confusion in the field with regard to using p53 gene therapy to treat tumor cells expressing wild-type p53. While the abstract states that “AdWTp53 vector exhibited a potent inhibitory effect on the growth of all [six] of human metastatic prostate cancer cells...,” the ensuing discussion paints a much muddier picture. For example, Srivastava states that:

Since several previous studies did not observe cell growth inhibitory effects of exogenous p53 in tumor cells that already contained endogenous wt p53,<sup>24,25</sup> the inhibitory effects of AdWTp53 on LNCaP cells containing endogenous wt p53 was unexpected. However, in agreement with the previous observations,<sup>18</sup> we also did not detect a growth inhibitory effect of AdWTp53 on breast cancer cells, MCF7 containing endogenous wt p53 (data not shown).

Page 845, second column.

A later statement makes it clear that Srivastava believes that there must be another p53 function that is being supplied by the exogenous wt p53 in these cells: “However, it is possible that some as yet unknown function of p53 is defective in LNCaP cells...” see page 847, second column. This suggests that one skilled in the art was directed away from the idea of clinically treating p53-positive cells with a p53 expression construct. Otherwise, the results in LNCaP cells would not need the justification afforded by this statement. Thus, reading Srivastava, one of skill in the art would not be led to believe that p53-positive cells could be treated with p53 gene therapy with any likelihood of success.

Turning next to the Katayose reference, this study employed adeno-p53 constructs to examine the susceptibility of various tumor cell lines (p53 null, p53 mutant, and p53 positive) to p53 gene therapy. Like Srivastava, Katayose demonstrates the confusion in the field with regard to treating p53-positive tumors with p53 gene therapy. The Katayose abstract states that “tumor cells that were null for p53 prior to infection...and tumor cells that expressed mutant endogenous p53 protein were more sensitive to AdWTp53 cytotoxicity than cells that contained the wild-type p53...” This rather non-committal statement is clarified by the last line of the introduction, which states that “these studies indicated that an adenovirus vector expressing wild-type p53 is markedly cytotoxic to tumor cells that have null or mutant p53 expression...” No mention is made of p53-positive cells. In addition, the last line of the abstract summarizes the authors conclusions: “These data suggest that endogenous p53 status is a determinant of AdWTp53-mediated cell killing of human tumors.” The clear inference is that only p53 null or p53 mutant tumor cells are killed by AdWTp53.

Katayose actually teaches away from treating p53 positive tumor cells with p53 expression vector. In the Discussion on page 896, first column, second paragraph, it is stated that “[t]here are several possible mechanisms by which high expression of wild-type p53 results in apoptosis in tumor cells devoid of p53 or expressing mutant p53, but not tumor or normal cells expressing wild-type p53.” Thus, Katayose is itself stating that expression of wild-type p53 would not be expected to effect apoptosis in a tumor that expresses wild-type p53.

The following additional comments also illuminate what the skilled artisan would take away from Katayose. “As shown in Fig. 3, *A* and *B*, infection of H-358 and MDA-MB231 cells [p53 null and mutant, respectively] with AdWTp53 completely inhibited cell growth.... In contrast, MCF-7 cells [p53 positive] continued to proliferate although at a slower rate than

control cells..." p. 892, second column. "It appears that cells that express wild-type p53 were 5-250 times more resistant to the AdWtp53-mediated inhibitory effect on cell growth when compared with cells expressing no p53 or mutant p53." p. 893, second column. "These results indicate that tumor cells null for p53 or expressing an endogenous mutant p53 undergo apoptosis following exposure to AdWtp53, whereas tumor cells or normal cells expressing wild-type p53 are resistant to apoptosis." p. 895, second column. "[O]verexpression of wild-type p53 induced programmed cell death (apoptosis) of tumor cells devoid of wild-type p53 or expressing endogenous mutant p53, but not in tumor or normal cells expressing wild-type p53." p. 896, first column. These passages clearly indicate that Katayose cannot be read as providing sufficient motivation for treating p53-positive cells. In fact, the reference suggests the opposite, that p53-positive cells are far less susceptible to such treatments.

*d) The Cajot et al. Reference is Flawed*

Another problem with the rejection is the Examiner's reliance on Cajot. Appellant asserts that these studies are unrelated to the actual therapy of human tumors, and are technically flawed. In particular, the Cajot reference is flawed in its use of the SV40 promoter in its p53 studies thereby skewing the results in such a way that the skilled artisan would not rely on the data.

In order to understand more fully the problems with Cajot, it is first necessary to explain the studies in some detail. Human lung cancer cells in culture were transfected with vectors containing either wild-type or mutant p53 under the control of the CMV promoter and the neomycin resistance gene under the control of the SV40 promoter. Cells were subjected to geneticin selection for 3-4 weeks. Thus, stable transformants were selected. Cajot concluded

that because few colonies were observed with wild-type p53 expressing clones there was an inhibitory effect on tumor cell growth by the p53 expressed in these cells.

What Cajot did not account for was the potent down-regulation of the SV40 promoter by p53. The ability of p53 to inhibit the SV40 promoter was well known in the art. Subler *et al.* (1992) (Exhibit L); Jackson *et al.* (1993) (Exhibit M); Perrem *et al.* (1995) (Exhibit N). The difference in the transfection efficiencies reported by Cajot between wild-type and mutant p53 can be explained by the fact that wild-type p53 inhibits the SV40 promoter, eliminating expression of the neomycin resistance gene, and thereby eliminating cells because of geneticin toxicity, not because of tumor inhibition.

Even Cajot bears out the notion that the remaining tumor clones survived because the p53 gene being expressed became mutated, thereby avoiding the SV40 down-regulation of neomycin: "In contrast, no normal-size transcript characteristic of exogenous p53 was detected in any of the wild-type p53 clones analyzed." p. 6957, second column. Also, in Figure 3, it should be noted that the tumorigenicity studies in nude mice were conducted with a clone that admittedly does not express a wild-type p53 product. In Figure 3, the only transfectant purported to be "wild-type" was the cell line designated "X833.W2." However, it is clear from Figure 1C and from the text that X833.W2 is not a wild-type clone at all – it expressed a mutant p53: "X833.W2 was shown by Western blot analysis to express what appears to be a mildly truncated form of the p53 protein." Page 6958, col. 2.

Another serious technical flaw exists with the *in vivo* studies in Cajot. The lung tumor cell lines were transfected with the p53 vector *ex vivo* (*in vitro*) and *then* injected into the nude

mice. Such an assay is not a true *in vivo* assay since one is not establishing a tumor *in vivo* first, and then treating the established tumor.

Where one employs an *ex vivo* assay such as was employed by Cajot, there is no test for the effects of the therapy on the tumor *in situ* in the patient's body. Many questions remain unaddressed by such an experiment – *e.g.*, is the vector capable of penetrating and entering the tumor cells *in situ*; does the therapy have an effect on the tumor mass when the tumor mass is actively growing in an animal (as opposed to mere cells in a test tube); is there sufficient distribution of the vector to cells of the tumor, and sufficient expression within those cells to effect a noticeable growth inhibitory effect; can the material pass through the extracellular matrix that comprises the tumor mass; are there extracellular proteins in the tumor milieu that might block uptake, *etc.*? Studying the effect of a gene such as p53 on cells *in vitro* tells one little about the ability of a gene to work as a tumor suppressor gene in the clinic, and would not be relevant to the pending claims, which are directed to direct administration to a tumor *in vivo*.

***e) There is No Reasonable Basis for Extrapolating from In Vitro and Animal Studies to Human Clinical Therapy***

The Examiner's combination of Srivastava, Katayose, and Cajot with Wills, Liu, and Zhang, as framed above, is improper. Appellant submits that the differences between *in vitro* and *in vivo* studies, and the differences between *in vivo* animal models and human clinical applications, especially when simultaneously making the leap from treating p53-negative to p53-positive cells, simply is not warranted.

The Examiner has the burden to show that the *in vitro* and animal studies are reasonably predictive of human clinical studies. The Examiner has not made this case. It is Appellant's position that the cited art that relates to p53-positive cells, which shows only *in vitro* data, cannot

be relied upon to predict what would happen in any *in vivo* context, much less in a human gene therapy context. The limitations of *in vitro* studies are manifest, and so well supported in the art that it is unnecessary to recount them here. In fact, the first Office Action addressed limitations on gene therapy, and specifically discussed the shortcomings of delivery and expression of transgenes *in vivo*:

The unpredictability of gene therapy and vector targeting is supported by the teachings of Culver *et al.*, Hodgson *et al.* and Miller *et al.* Culver *et al.*, reviewing gene therapy for cancer, conclude that the “primary factor hampering the widespread application of gene therapy to human disease is the lack of an efficient method for delivering genes *in situ*, and developing strategies to deliver genes to a sufficient number of tumor cells to induce complete tumor regression or restore genetic health remains a challenge” (page 178). Hodgson discusses the drawbacks of viral transduction and chemical transfection methods, and states that “[d]eveloping the techniques used in animal models, for therapeutic use in somatic cells, has not been straightforward” (pages 459-460). Miller *et al.* also review the types of vectors available for *in vivo* gene therapy, and conclude that “for all the long-term success as well as the widespread applicability of human gene therapy, there will have to be advances ... targeting strategies outlined in this review, which are currently only at the experimental level, will have to be translated into components of safe and highly efficient delivery systems” (page 198, column 1).

First Office Action, pages 5-6.

The art is replete with examples of cancer treatments that showed promise *in vitro* only to fail *in vivo*. For example, Planchon *et al.* (1992) (Exhibit O) showed that butyrate derivatives inhibited growth of breast cancer cell monolayers *in vitro*, but failed to affect the rate of tumor growth *in vivo*. Welters *et al.* (1999) (Exhibit P), in examining the effects on cisplatin in head & neck cancers, found a lack of correlation between studies on *in vitro* tumor cell lines and *in vivo* tumors. Vingerhoeds *et al.* (1996) (Exhibit Q) similarly compared the effects of doxorubicin on ovarian carcinoma cells and found that *in vitro* inhibition was not observed *in vivo*. Mourad *et al.* (1996) (Exhibit R) showed that high doses of vitamin A inhibited head & neck and lung cancers *in vitro*, but showed no similar effects *in vivo*. Liu *et al.* (2000) (Exhibit S) disclosed



that, *in vivo*, secretion of TGF- $\beta$  correlated with resistance to tumor therapy, while no correlation was observed *in vitro*. Finally, Johansson *et al.* (1991) (Exhibit T) demonstrated that a murine monoclonal antibody inhibited cancer cells *in vitro*, but that *in vivo* inhibition was limited to two days after inoculation into animals, hardly a clinically relevant situation.

Appellant also points out that the Katayose and Srivastava references, as discussed above, would not only fail to support the present rejection if considered, they actually *teach away*. Also as described above, the *in vitro* experiments with p53-positive cells described by Cajot are scientifically flawed. Furthermore, Cajot's tumorigenicity studies in nude mice used cells that were transfected with the p53 vector *ex vivo*; and the exogenous p53 expressed by the so-called "wild-type p53 transfection clone" that was injected into the mice was admittedly mutated.

The references cited by the Examiner do not refer to any human clinical findings, only to findings *in vitro* and in animal model systems (specifically the nude mouse model). In contrast, Appellant has provided scientific proof of human clinical data on the benefits of the wild-type p53 viral vector expression system in suppressing growth of HNSCC, thereby rendering the Examiner's arguments as null and void. The mere fact that animal and *in vitro* studies have been found reasonably predictive in the case of therapy of tumors bearing mutant p53 genes, there is no indication on this record that such studies would be reasonably predictive of success in treating animals having wild-type p53 tumors.

Thus, it is submitted that there is no basis for extrapolating from the *in vitro* studies of Srivastava, Katayose, and Cajot to the *in vivo* studies of Wills and Liu. This is based not only on the well established limitations of *in vitro* systems, but on the clear confusion in the field, as evidenced by Srivastava and Katayose.

Finally, the Examiner cites Zhang as teaching combination therapies. Even if Zhang does teach combination therapies, the combination of Srivastava, Cajot, Katayose, Wills, Liu, and Zhang still fails to teach or suggest a method of inhibiting growth of a tumor cell expressing wild-type p53 in a human subject. The Zhang reference is a review article on gene therapy for cancer. Zhang teaches only the replacement of p53 in cells lacking p53 or expressing a mutant p53; it does not teach p53 supplementation in cells expressing wild-type p53. With regard to p53 gene therapy, Zhang states, “research in this area is progressing rapidly and has demonstrated that the *restoration* of wild-type p53 function in tumour cells could be a very efficient approach to cancer therapy.” Zhang *et al.*, p. 494, col. 2 (emphasis added). This statement reinforces that the conventional thinking at the time was that Ad-p53 gene therapy would only be beneficial in tumors lacking a functional p53 molecule. There is no suggestion that a tumor cell having a functional p53 molecule may be treated using an expression construct that, itself, encodes a p53 molecule. Thus, the prior art does not combine to teach the treatment of tumor cells expressing wild-type p53 in a human subject.

In addition, for the reasons stated in section IX.E.5 below, Appellant submits that claims 4, 12, 13, 17, 18, 19 and 20, 26, 27, 29 and 30, 36, and 37 are separately patentable.

#### *f) Conclusion*

Appellant has provided numerous reasons as to why claims 1-14, 16-20, 26-32, 36, and 37 are not obvious in view of Srivastava, Cajot, Katayose, Wills, Liu, and Zhang. First, Srivastava is not available as prior art because it was published after the priority date of the present invention. Second, Dr. Clayman’s Rule 131 declaration avers conception of the present invention prior to August 11, 1995, the date of the earliest publication as between Srivastava and Katayose, and diligence to Appellant’s filing date in November, 1995. Thus, Srivastava and

Katayose are not properly citable against the present invention. Third, even if Srivastava and Katayose were citable against the present invention, they evidence confusion in the field with regard to the treatment of p53-positive tumor cells with vectors expressing p53 and, in fact, teach away from such an approach. Fourth, Cajot is scientifically flawed such that those skilled in the art would not rely on its teachings. Finally, because of the differences between *in vitro* and *in vivo* studies, and the differences between *in vivo* animal models and human clinical applications, especially when simultaneously making the leap from treating p53-negative to p53-positive cells, those of skill in the art would not have had a reasonable expectation of success based on the prior art.

For the reasons described above, claims 1-14, 16-20, 26-32, 36, and 37 are not obvious in view of Srivastava, Cajot, Katayose, Wills, Liu, and Zhang. Appellant requests that the Board reverse this rejection.

#### **E. Additional Arguments for the Separate Patentability of Specific Claims**

The arguments set forth above are intended to go to the novelty and non-obviousness of all of the pending claims. Appellant will now present additional arguments for the separate patentability of various dependent claims, which do not stand or fall together.

##### **1. The Liu *et al.* Reference**

As discussed above in Section IX.C.2, claims 1-9, 13, 14, 16-20, and 36 stand rejected as being anticipated by Liu. For the reasons set forth below, Appellant submits that claims 4, 13, 17, 18, 19-20, and 36 are separately patentable.

#### **Claim 4**

Claim 4 contains the limitation of a “benign” tumor cell. The Examiner has not shown where Liu teaches a method of inhibiting the growth of a benign tumor cell expressing wild-type p53 in a human subject. Therefore, a *prima facie* case for anticipation has not been established.

#### **Claim 13**

Claim 13 contains the limitation of administering the expression vector in a volume of about 3 ml to about 10 ml. The Examiner has not shown where Liu teaches this limitation. Therefore, a *prima facie* case for anticipation has not been established.

#### **Claim 17**

Claims 17 is directed to the “continuous perfusion” of a natural or artificial body cavity with a viral expression construct encoding a functional p53 polypeptide for inhibiting growth of a tumor cell expressing wild-type p53 in a human subject. The Examiner has not shown where Liu teaches “continuous perfusion.” Therefore, a *prima facie* case for anticipation has not been established.

#### **Claim 18**

Claim 18 contains the limitation of “an artificial body cavity resulting from tumor excision.” The Examiner failed to show where Liu teaches an artificial body cavity resulting from tumor excision. Thus, a *prima facie* case for anticipation has not been established.

#### **Claims 19 and 20**

Claims 19 and 20 are directed to a p53-encoding polynucleotide that is tagged so that expression of p53 from said expression vector can be detected. The Examiner failed to show where Liu teaches a p53 polynucleotide that is “tagged.” Thus, a *prima facie* case for anticipation has not been established.

### **Claim 36**

Claim 36 is directed to the treatment of a tumor located in a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen. The Examiner has not shown where Liu teaches inhibiting a tumor cell expressing wild-type p53 in any of these body cavities. Therefore, a *prima facie* case for anticipation has not been established.

### **2. The Clayman *et al.* Reference**

As discussed above in Section IX.C.3, claims 1-9, 13, 14, 16-20, and 36 stand rejected as being anticipated by Clayman. For the reasons set forth below, Appellant submits that claims 4, 13, 17, 18, 19-20, and 36 are separately patentable.

### **Claim 4**

Claim 4 contains the limitation of a “benign” tumor cell. The Examiner has not shown where Clayman teaches a method of inhibiting the growth of a benign tumor cell expressing wild-type p53 in a human subject. Therefore, a *prima facie* case for anticipation has not been established.

### **Claim 13**

Claim 13 contains the limitation of administering the expression vector in a volume of about 3 ml to about 10 ml. The Examiner has not shown where Clayman teaches this limitation. Therefore, a *prima facie* case for anticipation has not been established.

### **Claim 17**

Claims 17 is directed to the “continuous perfusion” of a natural or artificial body cavity with a viral expression construct encoding a functional p53 polypeptide for inhibiting growth of a tumor cell expressing wild-type p53 in a human subject. The Examiner has not shown where

Clayman teaches “continuous perfusion.” Therefore, a *prima facie* case for anticipation has not been established.

#### **Claim 18**

Claim 18 contains the limitation of “an artificial body cavity resulting from tumor excision.” The Examiner failed to show where Clayman teaches an artificial body cavity resulting from tumor excision.” Thus, a *prima facie* case for anticipation has not been established.

#### **Claims 19 and 20**

Claims 19 and 20 are directed to a p53-encoding polynucleotide that is tagged so that expression of p53 from said expression vector can be detected. The Examiner failed to show where Clayman teaches a p53 polynucleotide that is “tagged.” Thus, a *prima facie* case for anticipation has not been established.

#### **Claim 36**

Claim 36 is directed to the treatment of a tumor located in a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen. The Examiner has not shown where Clayman teaches inhibiting a tumor cell expressing wild-type p53 in any of these body cavities. Therefore, a *prima facie* case for anticipation has not been established.

### **3. The Roth *et al.* References**

As discussed above in Section IX.C.4, claims 1-14, 16-20, 26-32, 36, and 37 stand rejected as being anticipated by Roth *et al.* For the reasons set forth below, Appellant submits that claims 12, 19 and 20 are separately patentable.

#### **Claim 12**

Claim 12 is directed to the resection of a tumor cell expressing wild-type p53 in a human subject following at least a second administration of the expression vector, and an additional administration of the expression vector is effected subsequent to the resection. The Examiner failed to show where Roth teaches these limitations. Therefore, a *prima facie* case for anticipation has not been made.

#### **Claims 19 and 20**

Claims 19 and 20 are directed to a p53-encoding polynucleotide that is tagged so that expression of p53 from said expression vector can be detected. The Examiner failed to show where Roth teaches a p53 polynucleotide that is “tagged.” Thus, a *prima facie* case for anticipation has not been established.

#### **4. The Clayman *et al.*, Liu *et al.*, and Zhang *et al.* References**

As discussed above in Section IX.D.2, claims 1-14, 16-20, 26-32, 36, and 37 stand rejected as being obvious over Clayman *et al.* and Liu *et al.*, in view of Zhang *et al.* For the reasons set forth below, Appellant submits that claims 4, 11 and 26, 12, 13, 17, 18, 19-20, 27, 29-30, 36, and 37 are separately patentable.

#### **Claim 4**

Claim 4 contains the limitation of a “benign” tumor cell. The Examiner has not shown where the references teach or suggest a method of inhibiting the growth of a benign tumor cell expressing wild-type p53 in a human subject. Therefore, a *prima facie* case for obviousness has not been established.

#### **Claims 11 and 26**

Claims 11 and 26 contain the limitation of administering the expression construct to the tumor multiple times. The burden of showing a *prima facie* case of obviousness is on the

Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claims 11 and 26 are obvious in view of the prior art. The Examiner has not shown where the references teach or suggest multiple administrations of the expression vector. The Examiner has merely stated that it would be obvious. Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

### **Claim 12**

Claim 12 contains the limitation of the resection of a tumor cell expressing wild-type p53 in a human subject following at least a second administration of the expression vector, and an additional administration of the expression vector is effected subsequent to the resection. The Examiner failed to show where the references teach or suggest these limitations.

The Examiner asserts that Zhang teaches that gene therapy can successfully be combined with conventional therapies to enhance the treatment of cancer. However, the Examiner failed to show where Zhang teaches combining p53 gene therapy with tumor resection as described in claim 12.

Furthermore, Zhang states that “[i]n the near future, combinatorial approaches among gene therapies or gene therapy with conventional therapies such as chemotherapy, immunotherapy, radiotherapy, and surgery will be widely used, perhaps leading to the development of a more advanced biological therapy for cancer.” (p. 50, col. 2) Such a vague statement concerning the possible future of cancer therapy can hardly be said to provide a reasonable expectation of success in achieving the claimed invention.



Appellant, therefore, submits that a *prima facie* case for obviousness has not been established.

#### **Claim 13**

Claim 13 contains the limitation of administering the expression vector in a volume of about 3 ml to about 10 ml. The Examiner asserts that administering various volumes would be obvious, but provides no basis for this assertion. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has not met this burden. The Examiner failed to show where the references teach or suggest a volume of about 3 ml to about 10 ml or that there would be a reasonable expectation of success in using a volume of about 3 ml to about 10 ml. Therefore, a *prima facie* case for obviousness has not been established.

#### **Claim 17**

Claim 17 contains the limitation of “continuous perfusion” of a natural or artificial body cavity with a viral expression construct encoding a functional p53 polypeptide for inhibiting growth of a tumor cell expressing wild-type p53 in a human subject. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 17 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest “continuous perfusion.” Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

### **Claim 18**

Claim 18 contains the limitation of “an artificial body cavity resulting from tumor excision.” The Examiner argues that Zhang suggest combining gene therapy with conventional therapies such as surgery. Appellant asserts that the Examiner failed to show where the references teach or suggest an artificial body cavity resulting from tumor excision. A vague reference to the possible combination of gene therapy with “surgery” (see Zhang, p. 50, col. 2) does not teach or suggest the treatment of an artificial body cavity resulting from tumor excision, nor does it provide a reasonable expectation of success. Thus, a *prima facie* case for obviousness has not been established.

### **Claims 19 and 20**

Claims 19 and 20 contain the limitation of a p53-encoding polynucleotide that is tagged so that expression of p53 from said expression vector can be detected. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claims 19 and 20 are obvious in view of the prior art. The Examiner has not shown where the references teach or suggest a p53-encoding polynucleotide that is tagged. Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

### **Claim 27**

Claims 27 contains the limitation of multiple injections comprising about 0.1-0.5 ml volumes spaced about 1 cm apart. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is

obvious in light of the prior art. The Examiner has provided no evidence that claim 27 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest multiple injections comprising about 0.1-0.5 ml volumes spaced about 1 cm apart. The Examiner has merely stated that it would be obvious. Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

#### **Claims 29 and 30**

Claims 29 and 30 contain the limitation of contacting the tumor with a radiotherapeutic agent. The Examiner asserts that Zhang teaches that gene therapy can successfully be combined with conventional therapies, such as radiotherapy, to enhance the treatment of cancer. A vague reference to the possible combination of gene therapy with radiotherapy (see Zhang, p. 50, col. 2) does not provide the necessary motivation to combine or a reasonable expectation of success, particularly in the context of treating tumor cells expressing wild-type p53 in a human subject. Thus, a *prima facie* case for obviousness has not been established.

#### **Claim 36**

Claim 36 contains the limitation of a tumor located in a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 36 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest the body cavities recited in claim 36. The Examiner has merely

rejected the claim as obvious. Therefore, a *prima facie* case for obviousness has not been established.

#### **Claim 37**

Claim 37 contains the limitation of administering the expression construct to the tumor “at least six times within a two week treatment regimen.” The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 37 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest administering the expression construct to the tumor at least six times within a two week treatment regimen. The Examiner has merely stated that it would be obvious. Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

#### **5. The Srivastava *et al.*, Cajot *et al.*, Katayose *et al.*, Wills *et al.*, Liu *et al.*, and Zhang *et al.* References**

As discussed above in Section IX.D.3, claims 1-14, 16-20, 26-32, 36, and 37 stand rejected as being obvious in view of Srivastava *et al.*, Cajot *et al.*, Katayose *et al.*, Wills *et al.*, Liu *et al.*, and Zhang *et al.* For the reasons set forth below, Appellant submits that claims 4, 12, 13, 17, 18, 19-20, 27, 29-30, 36, and 37 are separately patentable.

#### **Claim 4**

Claim 4 contains the limitation of a “benign” tumor cell. The Examiner has not shown where the references teach or suggest a method of inhibiting the growth of a benign tumor cell expressing wild-type p53 in a human subject. Therefore, a *prima facie* case for obviousness has not been established.

### Claim 12

Claim 12 is directed to the resection of a tumor cell expressing wild-type p53 in a human subject following at least a second administration of the expression vector, and an additional administration of the expression vector is effected subsequent to the resection. The Examiner failed to show where the references teach or suggest these limitations.

The Examiner asserts that Wills and Zhang teach that gene therapy can successfully be combined with conventional therapies to enhance the treatment of cancer. However, the Examiner failed to show where Wills and Zhang teach combining p53 gene therapy with tumor resection as described in claim 12.

Furthermore, Zhang states that “[i]n the near future, combinatorial approaches among gene therapies or gene therapy with conventional therapies such as chemotherapy, immunotherapy, radiotherapy, and surgery will be widely used, perhaps leading to the development of a more advanced biological therapy for cancer.” (p. 50, col. 2) Such a vague statement concerning the possible future of cancer therapy can hardly be said to provide a reasonable expectation of success in achieving the claimed invention.

Appellant, therefore, submits that a *prima facie* case for obviousness has not been established.

### Claim 13

Claim 13 contains the limitation of administering the expression vector in a volume of about 3 ml to about 10 ml. The Examiner asserts that administering various volumes would be obvious, but provides no basis for this assertion. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has not met this burden.

The Examiner failed to show where the references teach or suggest a volume of about 3 ml to about 10 ml or that there would be a reasonable expectation of success in using a volume of about 3 ml to about 10 ml. Therefore, a *prima facie* case for obviousness has not been established.

#### **Claim 17**

Claim 17 is directed to the “continuous perfusion” of a natural or artificial body cavity with a viral expression construct encoding a functional p53 polypeptide for inhibiting growth of a tumor cell expressing wild-type p53 in a human subject. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 17 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest “continuous perfusion.” Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

#### **Claim 18**

Claim 18 contains the limitation of “an artificial body cavity resulting from tumor excision.” The Examiner argues that Wills and Zhang suggest combining gene therapy with conventional therapies. Appellant asserts that the Examiner failed to show where the references teach or suggest the treatment of an artificial body cavity resulting from tumor excision. A vague reference to the possible combination of gene therapy with “surgery” (see Zhang, p. 50, col. 2) does not teach or suggest the treatment of an artificial body cavity resulting from tumor

excision, nor does it provide a reasonable expectation of success. Thus, a *prima facie* case for obviousness has not been established.

#### **Claims 19 and 20**

Claims 19 and 20 are directed to a p53-encoding polynucleotide that is tagged so that expression of p53 from said expression vector can be detected. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claims 19 and 20 are obvious in view of the prior art. The Examiner has not shown where the references teach or suggest a p53-encoding polynucleotide that is tagged. Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

#### **Claim 27**

Claims 27 contains the limitation of multiple injections comprising about 0.1-0.5 ml volumes spaced about 1 cm apart. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 27 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest multiple injections comprising about 0.1-0.5 ml volumes spaced about 1 cm apart. The Examiner has merely stated that it would be obvious. Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success. Therefore, a *prima facie* case for obviousness has not been established.

### **Claims 29 and 30**

Claims 29 and 30 contain the limitation of contacting the tumor with a radiotherapeutic agent. The Examiner asserts that Wills and Zhang teach that gene therapy can successfully be combined with conventional therapies, such as radiotherapy, to enhance the treatment of cancer. A vague reference to the possible combination of gene therapy with radiotherapy (see Zhang, p. 50, col. 2; Wills, p. 1086, col. 2) does not provide the necessary motivation to combine or a reasonable expectation of success, particularly in the context of treating tumor cells expressing wild-type p53 in a human subject. Thus, a *prima facie* case for obviousness has not been established.

### **Claim 36**

Claim 36 is directed to the treatment of a tumor located in a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen. The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 36 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest the body cavities recited in claim 36. The Examiner has merely rejected the claim as obvious. Therefore, a *prima facie* case for obviousness has not been established.

### **Claim 37**

Claim 37 contains the limitation of administering the expression construct to the tumor "at least six times within a two week treatment regimen." The burden of showing a *prima facie* case of obviousness is on the Examiner, who must show evidence beyond merely stating that the



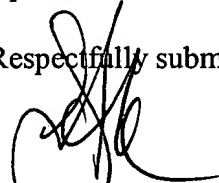
claimed invention is obvious in light of the prior art. The Examiner has provided no evidence that claim 37 is obvious in view of the prior art. The Examiner has not shown where the references teach or suggest administering the expression construct to the tumor at least six times within a two week treatment regimen. The Examiner states that Wills teaches the repetitive delivery of adenoviral vectors encoding wild-type p53. However, Wills appears to teach the delivery of the expression vector twice a week for four weeks, not at least six times within two weeks. Even if it were assumed that there was such a teaching, the Examiner has not shown that the prior art provides the motivation to combine the teachings or a reasonable expectation of success, particularly in the context of inhibiting the growth of a tumor cell expressing wild-type p53 in a human subject. Therefore, a *prima facie* case for obviousness has not been established.

#### X. CONCLUSION

It is respectfully submitted, in light of the above, that all of the pending claims are in condition for allowance. Appellant, therefore, requests that the Board overturn each of the pending grounds for rejection.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Respectfully submitted,



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Date: March 9, 2004

## APPENDIX 1

1. A method of inhibiting growth of a tumor cell expressing wild-type p53 in a human subject with a solid tumor comprising the steps of:
  - (a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and
  - (b) parenterally administering said viral expression construct to said subject, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth.
2. The method of claim 1 or 146, wherein said tumor is selected from the group consisting of a carcinoma, a glioma, a sarcoma, and a melanoma.
3. The method of claim 1 or 146, wherein said tumor cell is malignant.
4. The method of claim 1 or 146, wherein said tumor cell is benign.
5. The method of claim 1 or 146, wherein said tumor is a tumor of the lung, skin, prostate, liver, testes, bone, brain, colon, pancreas, head and neck, stomach, ovary, breast or bladder.
6. The method of claim 1 or 146, wherein said viral expression construct is selected from the group consisting of a retroviral vector, an adenoviral vector and an adeno-associated viral vector.

7. The method of claim 6, wherein said viral vector is a replication-deficient adenoviral vector.
8. The method of claim 7, wherein said replication-deficient adenoviral vector is lacking at least a portion of the E1-region.
9. The method of claim 8, wherein said promoter is a CMV IE promoter.
11. The method of claim 7, wherein the expression vector is administered to said tumor at least a second time.
12. The method of claim 11, wherein said tumor is resected following at least a second administration, and an additional administration is effected subsequent to said resection.
13. The method of claim 1, wherein said expression vector is administered in a volume of about 3 ml. to about 10 ml.
14. The method of claim 11, wherein the amount of adenovirus in each administration is between about  $10^7$  and  $10^{12}$  pfu.
16. The method of claim 1 or 146, wherein the expression construct is injected into a natural or artificial body cavity.
17. The method of claim 16, wherein said injection comprises continuous perfusion of said natural or artificial body cavity.
18. The method of claim 16, wherein said body cavity is an artificial body cavity resulting from tumor excision.
19. The method of claim 1 or 146, wherein the p53-encoding polynucleotide is tagged so that expression of p53 from said expression vector can be detected.

20. The method of claim 19, wherein the tag is a continuous epitope.
26. The method of claim 1 or 146, wherein said expression construct is administered to said tumor at least twice.
27. The method of claim 26, wherein said multiple injections comprise about 0.1-0.5 ml volumes spaced about 1 cm apart.
28. The method of claim 1 or 146, further comprising contacting said tumor with a DNA damaging agent.
29. The method of claim 28, wherein said DNA damaging agent is a radiotherapeutic agent.
30. The method of claim 29, wherein said radiotherapeutic agent is selected from the group consisting of  $\gamma$ -irradiation, x-irradiation, uv-irradiation and microwaves.
31. The method of claim 28, wherein said DNA damaging agent is a chemotherapeutic agent.
32. The method of claim 31, wherein said chemotherapeutic agent is selected from the group consisting of adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, verapamil, doxorubicin, podophyllotoxin and cisplatin.
36. The method of claim 1 or 146, wherein said tumor is located into a body cavity selected from the group consisting of the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, bladder interior and colon lumen.
37. The method of claim 11, wherein said expression construct is administered to said tumor at least six times within a two week treatment regimen.
146. A method of inducing apoptosis in a tumor cell expressing wild-type p53 in a human subject with a solid tumor comprising the steps of:

- (a) providing a viral expression construct comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a functional p53 polypeptide, wherein said polynucleotide is positioned sense to and under the control of said promoter; and
  - (b) parenterally administering said viral expression construct to said subject, the administration resulting in expression of said functional p53 polypeptide in cells of said tumor and inhibition of tumor cell growth.
147. The method of claim 1 or 146, wherein the expression construct is administered intravenously.
148. The method of claim 1 or 146, wherein the expression construct is administered by direct injection into the tumor.
149. The method of claim 1 or 146, wherein the expression construct is administered intraperitoneally.
150. The method of claim 1 or 146, wherein the expression construct is administered orthotopically.



## GROWTH SUPPRESSION OF SCC4N BY ADENOVIRUS WILD-TYPE p53

cDNA probe labeled by the random primer method in  $5 \times \text{SSC}$ - $5 \times$  Denhardt's solution-0.5% SDS-denatured salmon sperm DNA (20  $\mu\text{g}/\text{ml}$ ). The membrane was also stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA for RNA loading control. The relative quantities of p53 expressed were determined by densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

**Western Blot Analysis.** Total cell lysates were prepared by sonicating the cells 24-h postinfection in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) for 5 s. Fifty  $\mu\text{g}$  of protein from samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL membrane (Amersham). The membrane was blocked with Blotto/Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in phosphate-buffered saline) and probed with the primary antibodies, mouse anti-human p53 monoclonal antibody PAb1801 and mouse anti-human  $\beta$ -actin monoclonal antibody (Amersham), and the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN). The membrane was processed and developed as the manufacturer suggested.

**Immunohistochemical Analysis.** The infected cell monolayers were fixed with 3.8% formalin and treated with 3%  $\text{H}_2\text{O}_2$  in methanol for 5 min. Immunohistochemical staining was performed by using the Vectastain Elite kit (Vector, Burlingame, CA). The primary antibody used was the anti-p53 antibody PAb1801, and the secondary antibody was an avidin-labeled anti-mouse IgG (Vector). The biotinylated horseradish peroxidase avidin-biotin complex reagent was used to detect the antigen-antibody complex. Preabsorption controls were used in each immunostaining experiment. The cells were then counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO).

**Cell Growth Assay.** Cells were plated at a density of  $2 \times 10^4$  cells/ml in 6-well plates in triplicate. Cells were infected with either wild-type (Ad5CMV-p53) or replication-deficient adenovirus as a control. Cells were harvested every 2 days and counted; their viability was determined by trypan blue exclusion.

**Inhibition of Tumor Growth in Vivo.** The effect of Ad5CMV-p53 on established s.c. tumor nodules was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and use and for recombinant DNA research. Briefly, following induction of acepromazine/ketamine anesthesia, three separate s.c. flaps were elevated on each animal, and  $5 \times 10^6$  cells in 150  $\mu\text{l}$  of complete media were injected s.c. into each flap using a blunt needle; the cells were kept in the pocket with a horizontal mattress suture. Four animals were used for each cell line. After 4 days, the animals were reanesthetized, and the flaps were reelevated for the delivery of 100  $\mu\text{l}$  of: (a) Ad5CMV-p53 ( $10^8$  PFU) in the right anterior flap; (b) replication-defective virus ( $10^8$  PFU) in the right posterior flap; and (c) transport medium alone, in the left posterior flank. All injection sites had developed s.c. visual and palpable nodules before treatment was administered. Animals were observed daily and sacrificed on day 20. *In vivo* tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Following sacrifice, excised tumors were measured three dimensionally by microcalipers to determine tumor volume. A nonparametric Friedman's two-way analysis of variance test was used to test the significance of the difference between means of samples; the SPSS/PC+ software package (SPSS, Inc., Chicago, IL) was used.

## Results

**Adenoviral Infection of SCC4N Cells.** The conditions for optimal adenoviral transduction of Tu-138 and Tu-177 cells were determined by infecting these cells with adenovirus expressing the *Escherichia coli*  $\beta$ -gal gene. The transduction efficiency was assessed by counting the number of blue cells after X-gal staining. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used. Cells inoculated with a single dose of 100 MOI  $\beta$ -gal adenovirus exhibited 60% blue cells (Fig. 1A), and this was improved to 100% by multiple infections (data not shown). The transduction efficiency of this vector in SCC4N cells is quite different from that of other cell lines examined previously; HeLa, HepG2, LM2, and human non-small cell lung cancer cell lines

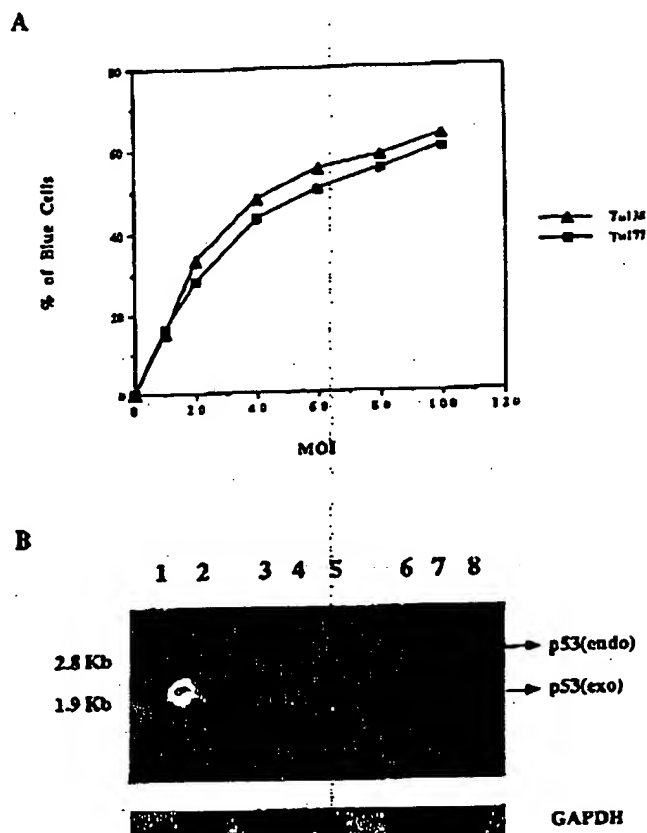


Fig. 1. A, transduction efficiency of SCC4N cell lines Tu-138 (▲) and Tu-177 (■). A recombinant  $\beta$ -gal adenovirus was used to infect the cells at different MOIs ranging from 10 to 100. The percentages of  $\beta$ -gal-positive cells were obtained from scoring 300 cells each on replicate dishes. B, expression of exogenous p53 mRNA 24 h after Ad5CMV-p53 infection. Lanes 1 and 2, 293 and K562 cells, respectively. Lanes 3 and 4, mock-infected Tu-138 and Tu-177 cells, respectively. Lanes 5 and 6, Tu-138 and Tu-177 cells infected with dl312. Lanes 7 and 8, Tu-138 and Tu-177 cells infected with Ad5CMV-p53.

showed 97 to 100% infection efficiencies after incubation with 30 to 50 MOI  $\beta$ -gal adenovirus (22).

**Expression of Exogenous p53 mRNA in Adenovirus-infected SCC4N Cells.** Two human SCC4N cell lines were chosen for this study; both cell lines Tu-138 and Tu-177 possess a mutated p53 gene (unpublished data). The recently created recombinant wild-type p53 adenovirus, Ad5CMV-p53, was used to infect Tu-138 and Tu-177 cells. Twenty-four h after infection, total RNA was isolated, and Northern blot analysis was performed. The transformed primary human embryonal kidney cell line 293 was used as a positive control because of its high level of expression of the p53 gene product, whereas K562, a lymphoblastoma cell line with a homozygous deletion of the p53 gene, was the negative control (Fig. 1B, Lanes 1 and 2, respectively). Due to unequal loading, only a fraction of the endogenous p53 mRNA was detected in the 293 cells (Fig. 1B, bottom panel). The levels of the 2.8-kilobase endogenous p53 mRNA detected in the samples isolated from mock-infected cells (Fig. 1B, Lanes 3 and 6) and from the cells infected with a replication-defective adenovirus, dl312 (Fig. 1B, Lanes 4 and 7), were similar. Up to 10-fold higher levels of exogenous 1.9-kilobase p53 mRNA were present in the cells infected with Ad5CMV-p53 (Fig. 1B, Lanes 5 and 8), indicating that the exogenous p53 cDNA was successfully transduced into these cells and efficiently transcribed. Interestingly, the level of endogenous p53 mRNA in these cells was 5-fold higher than in the experimental controls. Northern blots exhibited no evidence of Ad5CMV-p53 (DNA) contamination of RNA.

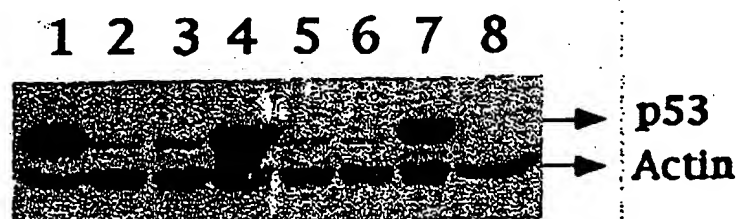
## GROWTH SUPPRESSION OF SCCRN BY ADENOVIRUS WILD-TYPE p53

**Expression of p53 Protein in Adenovirus-infected SCCRN Cells.** Western blot analysis was performed to compare the levels of p53 mRNA to the amount of p53 protein produced. A p53 band, recognized by monospecific anti-p53 antibody, PAb1801, was observed in cellular extracts isolated from all samples except K562 cells (Fig. 2A, Lane 8). Cell line 293 showed high levels of p53 protein (Fig. 2A, Lane 1). Samples isolated from mock-infected Tu-138 and Tu-177 cells exhibited low levels of p53 protein (Fig. 2A, Lanes 2 and 5). The level of p53 expression remained similar in those cells infected with the dl312 adenovirus (Fig. 2A, Lanes 3 and 6). The levels of p53 antigen detected in Ad5CMV-p53-infected cells were significantly higher than the levels of the endogenous mutated pro-

teins in both cell lines (Fig. 2A, Lanes 5 and 7). This result indicates that the exogenous p53 mRNA produced from cells infected with Ad5CMV-p53 is efficiently translated into immunoreactive p53 protein. Furthermore, immunohistochemical analysis of cells infected with Ad5CMV-p53 revealed the characteristic nuclear staining of p53 protein (Fig. 2B, right panel), whereas mock-infected cells failed to show similar staining despite the presence of the p53 protein in these cells (Fig. 2B, left panel). This inability to detect the protein may be attributable to the insensitivity of the assay.

**Effect of Exogenous p53 on SCCRN Cell Growth *in Vitro*.** Cells infected with control virus dl312 had growth rates similar to those of the mock-infected cells (Fig. 3), whereas growth of the Ad5CMV-

A



B

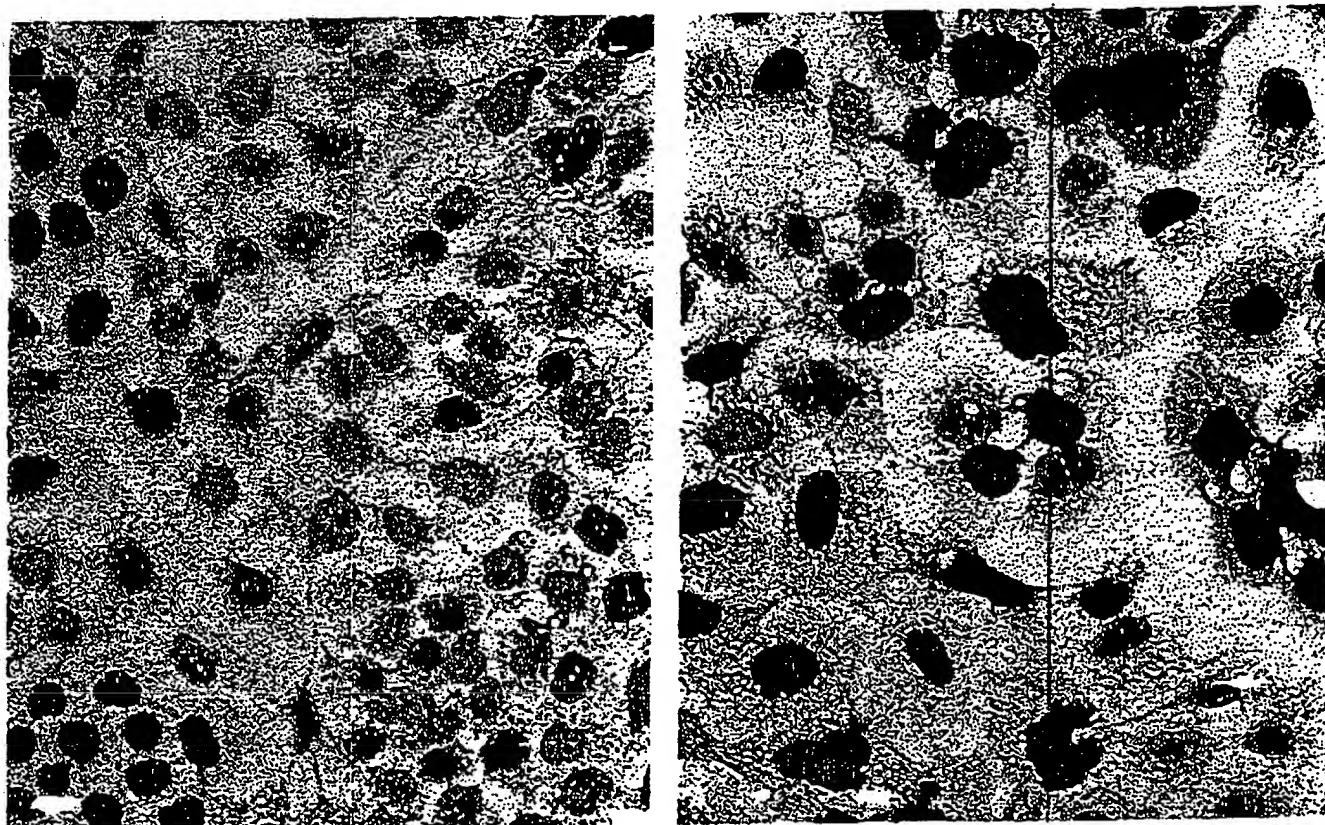


Fig. 2. A, Western blot analysis. Cellular extracts isolated from cells 24 h postinfection were subjected to SDS-polyacrylamide gel electrophoresis. Lanes 1 and 8, 293 and K562 cells, respectively. Lanes 2 and 5, mock-infected Tu-138 and Tu-177 cells. Lanes 3 and 6, Tu-138 and Tu-177 cells infected with dl312. Lanes 4 and 7, Tu-138 and Tu-177 cells infected with the Ad5CMV-p53. B, representative immunohistochemical staining of mock-infected Tu-138 cells (left) and Ad5CMV-p53-infected Tu-138 cells (right) 24-h postinfection.  $\times 250$ .



## GROWTH SUPPRESSION OF SCCN BY ADENOVIRUS WILD-TYPE p53

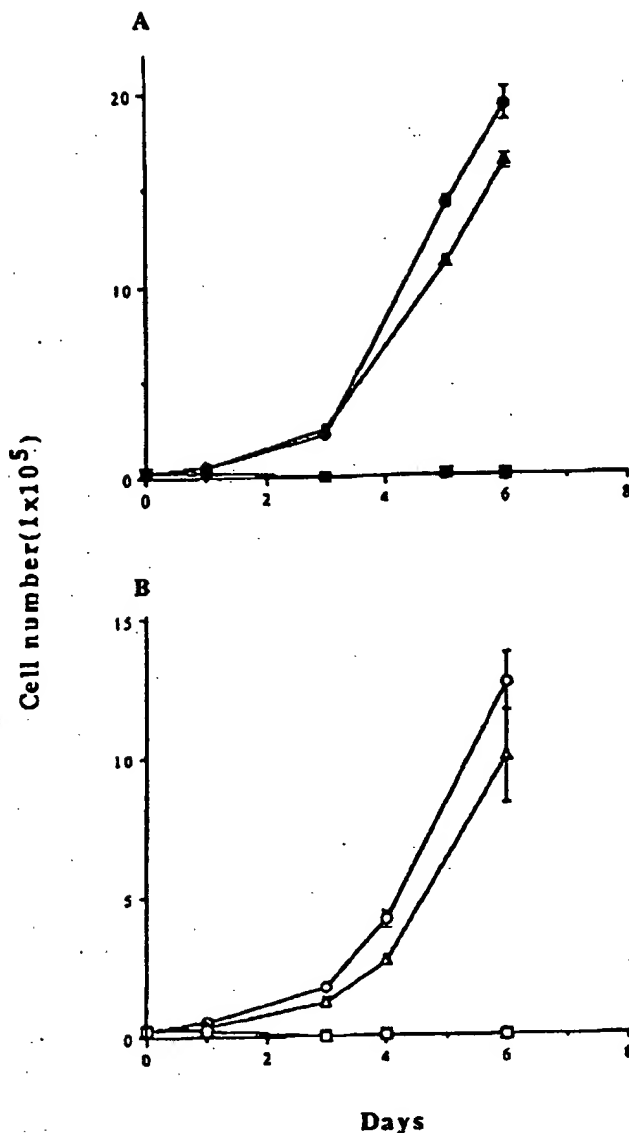


Fig. 3. Inhibition of SCCN cell growth *in vitro*. A, growth curve of mock-infected Tu-138 cells (●), d1312-infected cells (▲), and Ad5CMV-p53-infected cells (■). B, growth curve of mock-infected Tu-177 cells (○), d1312-infected cells (△), and Ad5CMV-p53-infected cells (□). At each indicated time point, three dishes of cells were trypsinized and counted. The mean of cell counts per triplicate wells following infection were plotted against the number of days since infection; bars, SEM.

p53-infected Tu-138 (Fig. 3A) and Tu-177 (Fig. 3B) cells was greatly suppressed. Twenty-four h after infection, an apparent morphological change occurred with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that constitute programmed cell death. The effect was more prominent for Tu-138 than for Tu-177 cells. Cells infected with the replication-defective adenovirus, d1312, demonstrated normal growth characteristics with no histomorphological abnormalities. Growth assays were reproducible in four repeated experiments.

**Inhibition of Tumor Growth *in Vivo*.** Seven animals were tested for each cell line. One animal in the Tu-177 group died following the second flap surgery and delivery of the therapeutic interventions, presumably due to profound anesthesia and subsequent mutilation by cage mates. Necropsy revealed no evidence of metastasis or systemic

effects. Fig. 4 shows representative Tu-138 (left) and Tu-177 recipients (right). Sizable tumors are apparent on both posterior flaps of the animals (i.e., the sites that did not receive Ad5CMV-p53). The lack of tumor progression is significant in the right anterior flaps of the animals which received Ad5CMV-p53 ( $P < .04$ ). That Tu-177 cells have a slower growth rate has been established previously in these animals.<sup>4</sup> Two animals in the Tu-177 group had complete clinical and pathological regression of their established s.c. tumor nodule. Two animals in the Tu-138 group were killed early because they were experiencing rapid growth and ulceration of the control tumor sites. All surgical sites had developed lesions of at least 6 mm<sup>3</sup> before intervention. The tumor volumes on necropsy are shown in Table 1.

### Discussion

Mutations or deletions of the p53 tumor suppressor gene are the most frequent genetic alterations reported in SCCN. Since the wild-type p53 gene is believed to be involved primarily in delivering antiproliferative signals that may be capable of antagonizing the growth-stimulatory signals propagated by oncogene products, the potential molecular therapeutic effect of this gene in SCCN deserves attention.

The rapid development in the field of gene therapy, including the creation of adenoviral vectors, has created an environment that is well suited for progress toward novel gene therapy of SCCN. Because of their natural tropism for aerodigestive tract epithelium, adenoviruses may be uniquely suitable for the transient delivery of genes to cancers in these epithelial tissues. The recombinant, replication-defective adenoviruses that have been developed for gene therapy are missing the entire E1a and part of the E1b regions and are, therefore, capable of propagating only in cells that can provide the E1 proteins *in trans*, such as the 293 cell line. In the past few years, recombinant adenoviruses have been extensively developed and used for *in vivo* gene therapy. The high transfer efficiency of adenoviral vectors over a broad range of hosts both *in vitro* and *in vivo* make them attractive vehicles for molecular therapy. Recently, a recombinant wild-type p53 adenoviral vector (Ad5CMV-p53) was generated. This provided us with an excellent candidate for investigation of the biological effects of the wild-type p53 gene product on SCCN cells bearing the mutated p53 gene. Using a  $\beta$ -gal recombinant adenovirus, the gene transfer efficiency of SCCN cells was established. Approximately 60% of SCCN cells were positive after X-gal staining. There appeared to be a linear correlation between the number of cells expressing the gene and the amount of viral particles used in the experiment. This result coincided with the efficiency obtained in cells infected with Ad5CMV-p53 after immunostaining by using a monoclonal anti-p53 antibody. Our observed transduction efficiency was lower than that achieved in other cell lines tested, including HeLa, HepG2, LM2, and the human non-small cell lung cancer cell lines. This discrepancy could be due to a host of factors, including receptor variations and differences in membrane characteristics among the cell lines. Additionally, the transduction efficiency of SCCN cells may have been underestimated by limitations of light microscopic analyses.

Ad5CMV-p53 mediated a high level of expression of the p53 gene in SCCN cells. Two p53 mRNA species were detected in the Ad5CMV-p53-infected cells. The high level of 1.9-kilobase mRNA was derived from the transduced p53 cDNA following infection with Ad5CMV-p53, indicating that the adenoviral vector is an efficient vehicle for gene delivery into human SCCN cells. Moreover, the levels of endogenous 2.8-kilobase mRNA were higher in the transduced cells than in the controls; presumably due to the effect of wild-type p53 gene product. This phenomenon of transcriptional

<sup>4</sup> Unpublished data.

## GROWTH SUPPRESSION OF SCCNH BY ADENOVIRUS WILD-TYPE p53

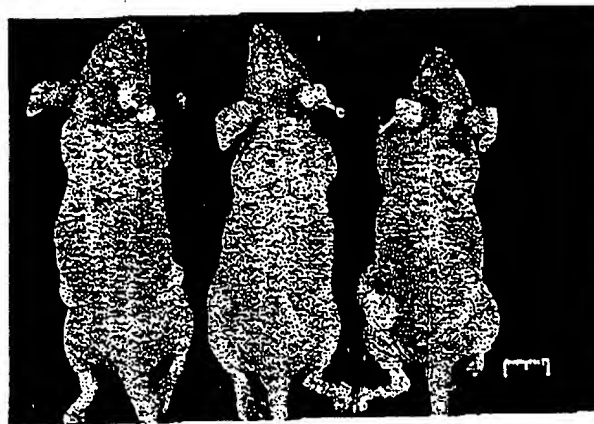


Fig. 4. Inhibition of SCCNH cell growth *in vivo*. Pictures of the representative nude mice studies for both Tu-138 (left) and Tu-177 (right) cell lines 20 days following therapeutic interventions. The right posterior flank received d1312, the left flank received transport medium alone, and the right anterior flap received Ad5CMV-p53, all 4 days following the establishment of a s.c. tumor.

Table 1 Effect of Ad5CMV-p53 on tumor growth in nude mice<sup>a</sup>

Treatment	Mean volume (mm <sup>3</sup> ± SEM)	
	Tu-138 (7)	Tu-177 (6)
Ad5CMV-p53	22.3 ± 14	13 ± 18
Ad5(d1312)	803 ± 300	533 ± 148
Medium	1297 ± 511	421 ± 143
Significance	P	P
p53 <sup>b</sup> :d1312	0.03	0.02
p53:medium	0.04	0.03

<sup>a</sup> The cells were injected s.c. at  $5 \times 10^6$  cells/flap. Tumor sizes were determined at day 20 after treatment. Numbers in parentheses, the number of animals evaluated.

<sup>b</sup> Ad5CMV-p53 is abbreviated as p53; d1312 is an abbreviation for Ad5(d1312).

autoregulation of the p53 gene has been well documented in murine cell lines in which the wild-type p53 can transactivate its own promoter and the mutant p53 fails to regulate the p53 promoter (24).

Due to the episomal property of adenoviral vectors, all the input DNA following infection with Ad5CMV-p53 is presumably degraded slowly throughout incubation. By using polymerase chain reaction-based detection techniques, DNA can be detected as late as 14 days postinfection (data not shown).

Western blot analysis demonstrated that there were few or no changes of p53 protein levels between mock- and replication-defective adenovirus-infected cells, whereas production of p53 protein was significant in Ad5CMV-p53-infected cells, suggesting that the exogenous p53 mRNA was efficiently translated. Time course protein expression studies have shown protein expression to peak 3 days postinfection and progressively decline to still detectable Western blotting levels on day 15 (22). Functionally, these SCCNH cells transduced with wild-type p53 gene exhibited significant inhibition of growth *in vitro* as compared to the mock-infected and replication-defective cells, thus clearly illustrating that these results were not mediated by the virus itself. The mechanism by which wild-type p53 protein inhibits growth *in vitro* may be related to arrest of the G<sub>1</sub> cell cycle (18), apoptosis (19, 20), or induction of another tumor suppressor gene such as WAF1/CIP1 (25). The induction of apoptosis is one of the several documented functions of wild-type p53. When Tu-138 and Tu-177 cells were infected with Ad5CMV-p53 at 100 plaque-forming units/cell, the characteristic apoptotic histomorphology, such as rounded-up cells and the formation of blebs, was apparent as early as 4 h after infection and was followed rapidly by cell death (data not

shown). However, the mechanism of growth suppression and cell death induced by Ad5CMV-p53 requires further investigation.

Encouraging results were also obtained in the nude mice studies. Tumor growth in the Ad5CMV-p53-infected cells was suppressed by at least 60 times more than in the experimental controls. These *in vivo* results confirmed the *in vitro* effects of Ad5CMV-p53 on human SCCNH cells, suggesting that the wild-type p53 protein mediates a potentially therapeutic effect. Although the *in vivo* studies are in their infancy, they nevertheless portend the development of a model for gene therapy in SCCNH that uses p53 adenovirus as a therapeutic intervention. Information derived from such studies could be expanded in the development of other novel molecular therapies that use adenoviral vectors, not only in SCCNH but in other human cancers. Several critical questions remain unanswered. How should the insult from antibodies that may arise in animals or patients following viral treatment be alleviated? How safe is this virus in humans? The results of the preliminary studies justify further investigation of *in vivo* animal models as well as mechanisms through which wild-type p53 regulates these *in vitro* and *in vivo* effects.

## References

1. Cancer Facts and Figures. Publication No. 98-425, M. No. 9808-LE. Washington, DC: American Cancer Society, 1990.
2. Thawley, S. E., and Panje, W. R. (eds.). Comprehensive Management of Head and Neck Tumors, Vol. 2, pp. 1158-1172. Philadelphia: W. B. Saunders, 1987.
3. van der Riet, P., Nawroz, H., Hruban, R. H., Corio, R., Tokino, K., Koch, W., and Sidransky, D. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. *Cancer Res*, 54: 1156-1158, 1994.
4. Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Fustan, I., Camou, S., and Shimizu, N. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. *Cancer Res*, 46: 414-416, 1986.
5. Somers, K. D., Cartwright, S. L., and Schechter, G. L. Amplification of the *int-2* gene in human head and neck squamous cell carcinomas. *Oncogene*, 5: 915-920, 1990.
6. Berenson, R., Yang, J., and Mickel, R. A. Frequent amplification of the *bcl-1* locus in head and neck squamous cell carcinomas. *Oncogene*, 4: 1111-1116, 1989.
7. Field, J. K., Spondidos, D. A., Stell, P. M., Vaughan, E. D., Evan, G. L., and Morre, J. P. Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma. *Oncogene*, 4: 1463-1468, 1989.
8. Rodriguez, N. K., Nowan, A., Smith, M. E. F., Kerr, I. B., Bodmer, W. F., Gannon, J. V., and Lane, D. P. p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci. USA*, 87: 7555-7559, 1990.
9. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. Genetic and immunoproteomic analysis of mutant p53 in human breast cancer cell lines. *Oncogene*, 5: 893-899, 1990.
10. Takahashi, T., Takahashi, T., Suzuki, H., Hida, T., Sekido, Y., Ariyoshi, Y., and Ueda, R. The p53 gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. *Cancer Res*, 52: 734-736, 1992.
11. Murali, R., Shalizi, M., Thilges, M., Kantarjian, H., Smith, L., Beran, M., Cork, A., Trujillo, J., Guterman, J., and Deisseroth, A. Rearrangement and expression of p53

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- in the chronic phase and blast crisis of chronic myelogenous leukemia. *Blood*, 75: 180-189, 1990.
12. Maestri, R., Dolcetti, R., Gasparotto, D., Dogliani, C., Pelucchi, S., Barzan, L., Grandi, E., and Boiocchi, M. High frequency of p53 gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. *Oncogene*, 7: 1159-1166, 1992.
  13. Chung, K. Y., Mukhopadhyay, T., Kim, J., Casson, A., Ro, J. Y., Goepfert, H., Hoeg, W. K., and Roth, J. A. Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. *53: 1676-1683, 1993.*
  14. Hoolstein, M., Sidransky, D., Vogelstein, B., and Harris, C. p53 mutations in human cancers. *Science (Washington DC)*, 253: 49-53, 1991.
  15. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science (Washington DC)*, 249: 912-915, 1990.
  16. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. W., and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA*, 87: 6166-6170, 1990.
  17. Cai, D. W., Mukhopadhyay, T., Liu, Y., Fujiwara, T., and Roth, J. A. Stable expression of the wild-type p53 gene in human lung cancer cells after retrovirus-mediated gene transfer. *Hum. Gen. Ther.*, 4: 617-624, 1993.
  18. Diller, L., Kessel, J., Nelson, C. E., Gryksa, M. A., Liwski, G., Gobhardt, M., Bressan, B., Ozark, M., Baker, S. J., and Vogelstein, B. p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.*, 10: 5772-5781, 1990.
  19. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordet, B., and Costa, J. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. USA*, 89: 4495-4499, 1992.
  20. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res.*, 53: 4129-4133, 1993.
  21. Kozarsky, K. F., and Wilson, J. M. Gene therapy: adenovirus vectors. *Curr. Opin. Gen. Dev.*, 3: 499-503, 1993.
  22. Zhang, W. W., Fang, X., Mazur, W., French, B. A., George, R. N., and Roth, J. A. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gen. Ther.*, 1: 1-10, 1994.
  23. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, 162: 156-159, 1987.
  24. Deffia, A., Wu, H., Reinkens, V., and Lozano, G. The tumor suppressor p53 regulates its own transcription. *Mol. Cell. Biol.*, 13: 3415-3423, 1993.
  25. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., et al. WAF1/CIP1 is induced in p53-mediated G<sub>1</sub> arrest and apoptosis. *Cancer Res.*, 54: 1169-1174, 1994.

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## Advances in Brief

Growth Suppression of Human Head and Neck Cancer Cells by the Introduction of a Wild-Type *p53* Gene via a Recombinant Adenovirus<sup>1</sup>Ta-Jen Liu, Wei-Wei Zhang, Dorothy L. Taylor, Jack A. Roth, Helmuth Goepfert, and Gary L. Clayman<sup>2</sup>

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## Abstract

Mutations of the *p53* gene constitute one of the most frequent genetic alterations in squamous cell carcinoma of the head and neck (SCCHN). In this study, we introduced wild-type *p53* into two separate SCCHN cell lines via a recombinant adenoviral vector, Ad5CMV-*p53*. Northern blotting showed that following infection by the wild-type *p53* adenovirus (Ad5CMV-*p53*), cells produced up to 10-fold higher levels of exogenous *p53* mRNA than cells treated with vector only (without *p53*). Western blotting showed that the increased levels of *p53* protein produced in the Ad5CMV-*p53*-infected cells were a reflection of *p53* mRNA expression. *In vitro* growth assays revealed growth arrest following Ad5CMV-*p53* infection as well as cell morphological changes consistent with apoptosis. *In vivo* studies in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral infiltration of Ad5CMV-*p53*. These data suggest that Ad5CMV-*p53* may be further developed as a potential novel therapeutic agent for SCCHN since introduction of wild-type *p53* into SCCHN cell lines attenuates their replication and tumor growth.

## Introduction

Patients with SCCHN<sup>3</sup> are afflicted with a disease process that often has profound effects on speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients has remained unchanged at approximately 45% for nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Treatment failures among these patients remain local and regional; only 10–15% of patients with the disease die of distant metastasis alone (2).

Although we have gained in understanding of the molecular events in the initiation and progression of SCCHN, they continue to require intensive investigation. A recent study identifying loss of heterozygosity of chromosome 9p21–22 as the most frequent genetic alteration in SCCHN suggested that this may be an early event in progression toward this neoplasm (3). Additionally, amplification and/or overexpression of cellular and nuclear oncogenes, such as *c-erbB-1* (4), *int-2* (5), *bcl-1* (6) and *c-myc* (7), have been documented in these cancers. The tumor suppressor gene *p53* has been the subject of immense

interest and investigation in recent years. Alterations in the *p53* gene, including deletion, insertion, and point mutation, are the most frequent genetic events in many different carcinomas, such as those of the colon (8), breast (9), and lung (10), as well as soft-tissue sarcomas and leukemias (11). Several investigators have demonstrated the high frequency of *p53* gene alterations in SCCHN (12, 13).

There is considerable evidence implicating mutations of the *p53* gene in the etiology of many human cancers (14). Reports have demonstrated that growth of several different human cancer cell lines, including representatives of colon cancer (15), glioblastoma (16), breast cancer (17), and osteosarcoma (18), can be functionally suppressed by DNA transfection or retrovirus-mediated transfer of the wild-type *p53* gene. This gene may have an important role not only in cell growth but in apoptosis (programmed cell death). Induction of exogenous expression of wild-type *p53* has been shown to induce apoptosis in colon cancer cell lines (19) and in human lung cancer spheroids (20).

The adenoviral vector has emerged as a leading candidate for *in vivo* gene therapy in the past few years. It enjoys an advantage over traditional DNA transfection and retroviral transfer in its high efficiency of transferring potentially therapeutic genes into a wide range of host cells (21). The recently created adenoviral vector containing wild-type *p53* (Ad5CMV-*p53*; Ref. 22) provides us with an attractive delivery system to investigate the effect of exogenous wild-type *p53* on SCCHN cell lines both *in vitro* and *in vivo*. The outcome of this study indicates that further development of the *p53* adenovirus or other novel molecular therapies for SCCHN is warranted.

## Materials and Methods

**Cell Lines and Culture Conditions.** Human SCCHN cell lines Tu-138 and Tu-177 were both established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center. Tu-138 and Tu-177 were established from a gingivo-labial moderately differentiated squamous carcinoma and a poorly differentiated squamous carcinoma of the larynx, respectively. Both cell lines were developed via primary explant technique and are cytokeratin positive and tumorigenic in athymic nude and SCID mice. These cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FBS with penicillin/streptomycin.

**Recombinant Adenovirus Preparation and Infection.** The recombinant *p53* adenovirus (Ad5CMV-*p53*; Ref. 22) contains the CMV promoter, wild-type *p53* cDNA, and SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified Ad5. Viral stocks were propagated in 293 cells. Cells were harvested 36–40 h after infection, pelleted, resuspended in phosphate-buffered saline, and lysed; cell debris was removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed, aliquoted, and stored at –80°C. Infection was carried out by the addition of the virus to the DMEM/F12 medium and 2% FBS to the cell monolayers. The cells were incubated at 37°C for 60 min with constant agitation. Then complete medium (DMEM/F12–10% FBS) was added, and the cells were incubated at 37°C for the desired length of time.

**Northern Blot Analysis.** Total RNA was isolated by the acid-guanidinium thiocyanate method of Chomczynski and Sacchi (23). Northern analyses were performed on 20 µg of total RNA. The membrane was hybridized with a *p53*

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<sup>3</sup>The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12 medium; FBS, fetal bovine serum; Ad5, adenovirus 5; CMV, cytomegalovirus; Ad5CMV-*p53*, wild-type *p53* adenovirus; cDNA, complementary DNA; MOL, multiplicity of infection; SDS, sodium dodecyl sulfate; β-gal, β-galactosidase; dl312, replication-defective adenovirus; PFU, plaque forming units.

# **In Vivo Molecular Therapy with p53 Adenovirus for Microscopic Residual Head and Neck Squamous Carcinoma<sup>1</sup>**

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## **Abstract**

Developing gene therapy strategies may allow contemporary medicine to reassess its management of solid malignancies. We have demonstrated previously that the wild-type p53 adenovirus (Ad5CMV-p53) suppressed the growth of established tumors of the head and neck. In this paper we develop a microscopic residual model which mimics the postsurgical environment of head and neck cancer patients with advanced disease. Using this squamous cell carcinoma of the head and neck model, we prevented the establishment of tumors in nude mice in which tumor cells had been s.c. implanted by transiently introducing exogenous wild-type p53 via an adenoviral vector 2 days following tumor cell implantation. These effects were vector dose dependent but independent on the endogenous wild-type or mutated p53 status of the cells. Importantly, karyotypically normal and nontumorigenic fibroblast cell lines are inert to the p53 adenovirus treatment. These results pave the ground work for further development of molecular therapy for head and neck cancer and other solid malignancies.

## **Introduction**

Patients with SCCHN<sup>3</sup> are afflicted with a disease process that often has profound effects upon speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients, approximately 50%, has remained unchanged for the nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Recurrences among these patients remain predominantly local and regional; approximately only 10% of patients die of distant metastasis alone (2, 3). In patients with SCCHN, the pathological findings of extracapsular invasion, neurotropism, and microscopic residual disease necessitate adjunctive therapy and predict aggressive local-regional disease. Moreover, these factors can usually be predicted prior to surgical intervention.

In head and neck cancer, direct gene transfer to microscopic residual carcinoma may not be technically difficult. When the primary tumor is removed, the tumor milieu is readily accessible for molecular therapy and is the most likely pathway of lymphatic spread when the regional lymphatic dissection is performed. Therefore, novel means of addressing assumed microscopic residual disease using direct transfer of genes that encode toxic products, specific tumor suppressor genes,

or genes that induce products that specifically promote tumor cell death and spare nonmalignant cells may provide desperately needed improvement in local-regional control among these patients and thus be an important approach to cessating these malignancies. In addition, several other solid malignancies possess the same dilemma, and therefore the model of SCCHN may provide insight into cancers of several other organ systems.

We believe that promising new therapies for SCCHN are interventions at the molecular level, and adenovirus-mediated gene transfer is our clinical method of choice for such intervention. Adenoviruses have a known tropism for the epithelium of the aerodigestive tract and are linked only to minor disease in humans (4). Moreover, in contrast to retroviruses, they are capable of transferring genes to nonproliferating cells, which appears preferable because of the heterogeneity of cell cycling within the tumor microenvironment (5). Finally, the transient nature of gene expression after adenoviral gene delivery allows selection of a molecular intervention that will provide the desired outcome (tumor cell death in cancer) without long-term integration of the recombinant molecular therapy into bystander cells and the potential ramifications thereof.

## **Materials and Methods**

**Cell Lines and Culture Conditions.** Human SCCHN cell lines Tu-138, Tu-177, MDA 686-LN, and MDA 886 were all established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center, and had been characterized previously (6, 7). These cells were grown in DMEM (DMEM/Ham's F-12) supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin.

**Recombinant Adenovirus Preparation and Infection, Cell Growth Assay, and Western Blot Analysis.** All the procedures have been described previously (8). Cell growth assays were all performed in triplicate.

**In Vivo Transduction with  $\beta$ -Galactosidase Adenovirus.** X-Gal staining of tissue specimens were performed on ornithine carbamyl transferase [Tissue Tek O. C. T. Compound<sup>®</sup> (Miles, Elkhart, IN)] frozen tissue sections to determine transduction efficiency. Eight- $\mu$ m-thick specimens were washed in cold PBS and fixed in 0.5% glutaraldehyde at room temperature for 5 min. Slides were then washed twice with 4°C PBS and incubated for 4 h in X-Gal solution [1.3 mM MgCl<sub>2</sub>, 15 mM NaCl, 44 mM Hepes buffer (pH 7.4); 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 2% X-Gal in *N,N*-dimethylformamide]. Slides were counterstained with H & E.

**Immunohistochemical Analysis.** Formalin-fixed paraffin-embedded *in vivo* animal experimental tissues were cut at 4–5  $\mu$ m, dried at 60°C, deparaffinized, and hydrated with distilled water. Sections were then treated with 0.5% saponin in distilled water and rinsed in several changes of distilled water; endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol, followed by rinsing in several changes of distilled water. Sections were microwave-irradiated in distilled water for 3 min using a Sharp Model R9H81 microwave oven operating at a frequency of 2450 MHz at 700 W. After cooling, sections were washed in several changes of distilled water and placed in PBS; immunochemical studies were performed by using the avidin-biotin-peroxidase complex method of Hsu *et al.* (9) in the following manner: sections were blocked with normal horse serum and incubated overnight at 4°C with

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<sup>3</sup> The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; Ad5CMV-p53, wild-type p53 adenovirus; CMV, cytomegalovirus; Ad5, adenovirus serotype 5; PFU, plaque-forming units; X-Gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside.

rabbit antihuman *p53* polyclonal antibody, clone OM-1, 1:80 (Signet Laboratories, Denham, MA). An anti-rabbit IgG Elite kit (Vector Laboratories, Burlingame, CA) was then used to apply biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complexes which were incubated for 45 min each. The immunostaining reaction was visualized by using 0.5% dimethylaminoazobenzene in PBS containing 0.01% hydrogen peroxide (pH 7.6), counterstained with 0.01% toluidine blue, dehydrated, cleared, and mounted in Permount. To verify the specificity of the immunostaining reaction, immunoperoxidase staining was performed (using the same method as on test samples) on a known positive cytospin of a tissue culture of a squamous carcinoma cell line as well as on a negative rabbit monoclonal antibody control.

**Inhibition of Tumor Growth *in Vivo*.** The effect of Ad5CMV-*p53* on a microscopic disease model of SCCN was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and utilization and the Biosafety Committee for recombinant DNA research. Briefly, nude female mice (aged 4–7 weeks) were anesthetized with i.p. ketamine/acepromazine (70 mg/kg of body weight) (Parke-Davis, Morris Plains, NJ). After their bodies were prepared with alcohol wipes, incisions were made in the dorsal flanks and three s.c. flaps were elevated with sharp dissection. Sterile pipette dispensers were used to introduce the desired number of tumor cells in 100  $\mu$ l of culture medium into the flap, which was sealed with a horizontal mattress suture. Forty-eight h following tumor-cell delivery, the animals were reanesthetized and the sutures removed. The flap was infected with Ad5CMV-*p53*, dl312,  $\beta$ -galactosidase adenovirus, or PBS alone (mock infection) by pipetting in 100- $\mu$ l increments; the flap was then resealed with a horizontal mattress suture. The PFU of the inoculant was increased in log increments with animals serving as their own controls as well as single-flap models on animals. The animals were observed daily for tumor development and killed in cases of excessive tumor burden or after 12 weeks of observation. All surgical sites were evaluated pathologically as well as by necropsy analysis for systemic toxicity.

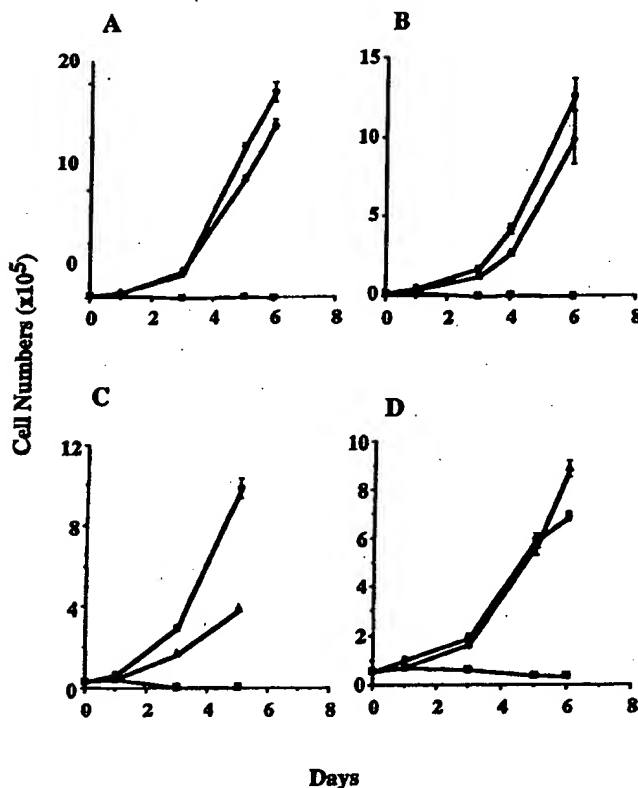
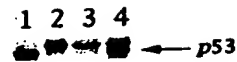


Fig. 1. Composite growth curve of four SCCN cell lines. A, Tu-138; B, Tu-177; C, MDA 686-LN; D, MDA 886. Mock infected cells (●), dl312-infected cells (▲), and Ad5CMV-*p53* infected cells (■). The mean of cell counts per triplicate wells following infection were plotted against the number of days post-infection; bars, SEM.

A



B

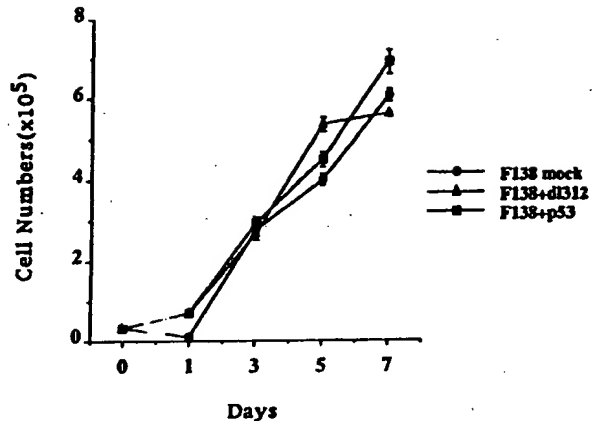


Fig. 2. Expression of exogenous *p53* protein in adenovirus infected normal fibroblasts and its effect on growth rate. A, Western blot analysis. Cellular extracts isolated from cells 24-h postinfection were subjected to SDS-PAGE. Lane 1, Tu-138 infected with the Ad5CMV-*p53*; Lane 2, mock-infection of Fibroblast-138; Lane 3, Fibroblast-138 cells infected with the replication-defective adenovirus, dl312; Lane 4, Fibroblast-138 cells infected with the Ad5CMV-*p53*. B, Growth curve of normal fibroblast cell line. Mock infected cells (●), dl312 infected cells (▲), and Ad5CMV-*p53*-infected cells (■).

## Results

**Effect of Exogenous *p53* on SCCN Cell Growth *in Vitro*.** We described previously the *in vitro* inhibition of cell growth by Ad5CMV-*p53* in SCCN cell lines with endogenously mutated *p53* (8). We therefore sought to determine whether SCCN cell lines with endogenous wild-type *p53* would be similarly affected. We also investigated the effect of Ad5CMV-*p53* on nonmalignant fibroblasts.

Four human SCCN cell lines were chosen for this study: Tu-138 and Tu-177, which possess a mutated *p53* gene, and MDA 686-LN and MDA 886, which are homozygous for the wild-type *p53* gene.<sup>4</sup> A fibroblast cell line derived from normal fibroblast outgrowth, which is karyotypically normal and nontumorigenic, was used as a nonmalignant control cell line. Cells infected with the control virus, dl312, had growth rates similar to those of the mock-infected cells, whereas the growth of tumor cells infected with Ad5CMV-*p53* was significantly suppressed (Fig. 1). Twenty-four–48 h after infection, an apparent morphological change occurred in all tumor cells, with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that constitute programmed cell death. The effect occurred earlier in cells with endogenous mutated *p53* than it did in those cells with wild-type *p53*. Cells infected with replication-defective adenovirus, dl312, demonstrated normal growth characteristics with no histomorphological abnormalities. Growth assays were reproducible in four repeated experiments.

<sup>4</sup> G. L. Clayman, unpublished data.



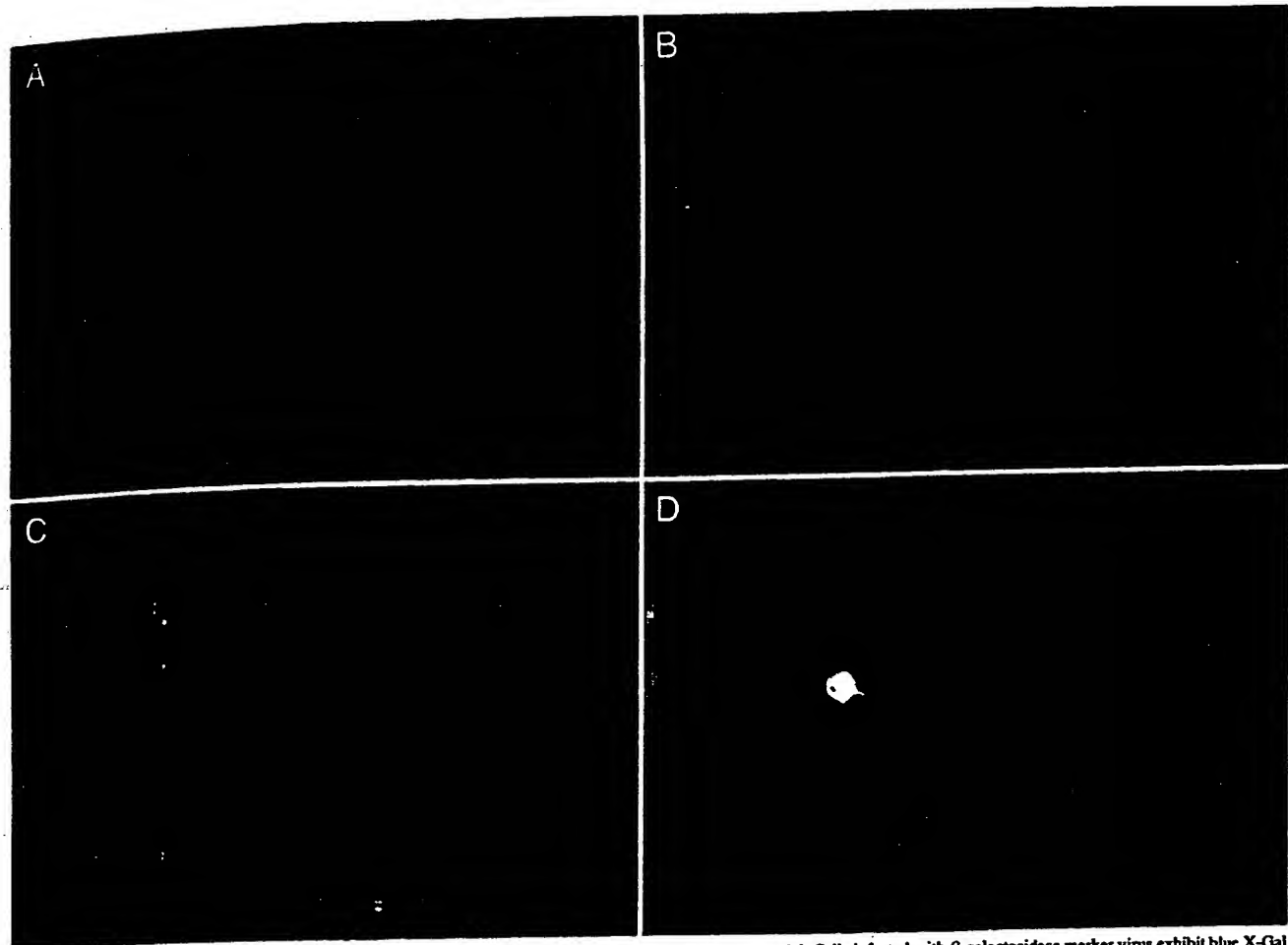


Fig. 3. Dose-response relationship in *in vivo* infection experiments in the microscopic residual disease flap model. Cells infected with  $\beta$ -galactosidase marker virus exhibit blue X-Gal staining. A, mock infection; B,  $10^7$  PFU viral particles; C,  $10^8$  PFU viral particles; D,  $10^9$  PFU viral particles. A clear dose response is evident. Histological inflammation and edema increase with increasing viral titers. Magnification, 63x.

**Expression of Exogenous p53 Protein in Adenovirus Infected Normal Fibroblasts and its Effect on Growth Rate.** Additionally, we investigated the effect of the Ad5CMV-p53 on karyotypically normal and nontumorigenic fibroblast cell lines. These cells were isolated during the establishment of primary tumor cell lines. Twenty-four h after infection, Western blot analysis was performed to compare the levels of protein produced by the different infected cell types. A p53 band, recognized by the monospecific anti-p53 antibody, PAh1801, was observed in cellular extracts isolated from all samples infected with the Ad5CMV-p53 (Fig. 2A; Lanes 1 and 4). As has been shown previously (8), cell line Tu-138 infected with the p53 adenovirus showed high levels of p53 protein following transduction and served as a control (Fig. 2A; Lane 1). The level of p53 expression remained similar in both mock-infected and dl312-infected cells (Fig. 2A; Lanes 2 and 3). The Ad5CMV-p53-infected fibroblasts showed higher levels of p53 protein than did the control cells (Fig. 2A; Lane 4). This result indicates that the p53 gene is efficiently translated into normal fibroblasts infected with Ad5CMV-p53 as evidenced by production of immunoreactive p53 protein. The protein expression and transduction efficiency of cytopins of Ad5CMV-p53 infected fibroblasts were verified by immunohistochemical analysis (data not shown). This fibroblast cell line exhibited normal growth rate and morphology independent of the intervention (mock, replication-defective virus, or Ad5CMV-p53) (Fig. 2B). These experiments were

repeated twice and also verified in other normal human fibroblast cell lines.

***In Vivo* Transduction Efficiency.** To measure the efficiency of gene transfer *in vivo*, we resected the s.c. flap site 72 h following molecular or control intervention. Dose-response experiments with the adenovirus  $\beta$ -galactosidase-marker vector demonstrate dose-response transduction efficiency in this model (Fig. 3). This was confirmed with immunohistochemical analysis 4 days following infection with Ad5CMV-p53 (Fig. 4). Both groups of experiments exhibited an *in vivo* dose response which had been described previously *in vitro* by us and others (8). In no instances did doses of virus exceeding  $10^{10}$ -PFU effect expression of p53 in other organ systems including brain, liver, lung, heart, abdominal visceral organs, and skin (data not shown). These experiments illustrated a dose-response relationship between viral titer and transduction efficiency as well as the possibility of achieving extensive transient expression of the transduced gene within the desired surgical model field.

**Suppression of Tumor Growth *in Vivo*.** We designed our initial experiments to determine whether *in vivo* Ad5CMV-p53 mediated gene transfer would affect the establishment or growth of SCCNH cells implanted into a s.c. flap. We created a microscopic residual disease model. In this model, three s.c. flaps were elevated on athymic nude female mice, and  $2.5 \times 10^6$  of tumor cells were seeded by pipetting. Instead of allowing the tumor cells to form nodules

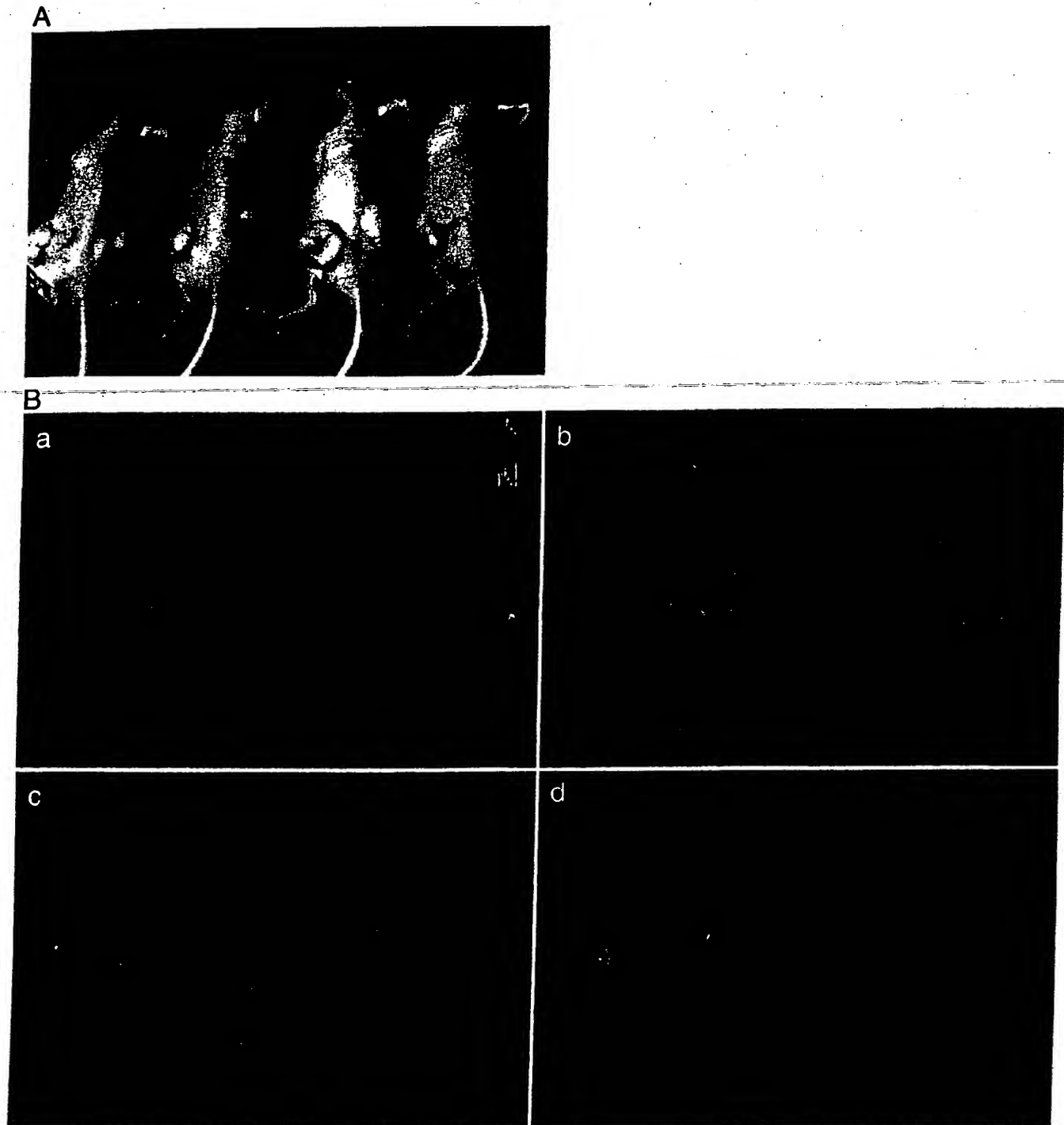


Fig. 4. A, Suppression of tumor establishment in a microscopic residual disease model of SOCHN. Nude female mice implanted with Tu-138 cell lines and delivered 48 h later with 100  $\mu$ l of AdSCMV-p53 ( $10^8$  PFU) in the right anterior flap d1312 (replication defective adenovirus)  $10^8$  PFU in the right posterior flap and transport medium (PBS) alone in the left posterior flap. Animals were observed daily and sacrificed at 12 weeks or earlier if excessive tumor burden developed. No gross or histological evidence of tumor in the AdSCMV-p53 flap site was found. Photograph taken at 8 weeks. B, *In vivo* infectivity of AdSCMV-p53 in the microscopic residual disease flap model. The AdSCMV-p53 was pipetted into the s.c. flap 48 h following tumor cell line delivery. A representative experiment of the wild-type *p53* SCCHN cell line MDA 686-LN is shown. Panel a, mock infection showing lack of immunostaining in the wild-type *p53* cell line; Panel b,  $10^7$  PFU of AdSCMV-p53; Panel c,  $10^8$  PFU of AdSCMV-p53; Panel d,  $10^9$  PFU of AdSCMV-p53. Immunostaining was performed using the polyclonal rabbit antihuman antibody OM1 (Sigma Laboratories) using the avidin-biotin method. A clear dose-response relationship is shown. No viable tumor was found in multiple histological sections of  $10^8$  or  $10^9$  PFU AdSCMV-p53. Magnification 100x.



occurring in 4 days), we delivered our single dose of *p53* intervention at 48 h following tumor cells seeding. In this model, although no gross tumors were present, microscopic tumor cells were within the surgical site mimicking the clinical dilemma of surgical excision of all gross tumor. The development of tumors was directly related to the number of tumor cells, the time allotted for implantation, and the dose of Ad5CMV-*p53*. Of the mice which received microscopically implanted tumor cells ( $2.5 \times 10^6$ ) and were treated with Ad5CMV-*p53* at  $10^8$  PFU or greater, only two mice developed tumors, both of which were implanted with the wild-type *p53* cell line (MDA 886-LN). All other cell lines exhibited absence of tumor development (Table 1). These experiments clearly indicate that the growth of microscopic tumor cells can be effectively suppressed *in vivo* if exposed to the Ad5CMV-*p53* (Fig. 4A). Tumor formation was evaluated at the end of a 12-week period (earlier animal sacrifice in circumstances of excessive tumor burden) by gross and histological analysis of the surgical sites. The data of tumor establishment is summarized in Table 1, and a representative experiment is shown in Fig. 4A.

Immunohistochemical analysis was performed on the tumor sections of experimental animals. A representative experiment of the SCCN cell line MDA 686-LN is shown (Fig. 4B). This cell line possesses the wild-type endogenous *p53* gene. Lack of significant basal immunostaining with the viable tumor of MDA 686-LN (mock-infection) is seen in Panel A. Panel B (Ad5CMV-*p53* at  $10^7$  PFU) shows peripheral tumor necrosis with immunostaining in the more central portion of the tumor. Panel C (Ad5CMV-*p53* at  $10^8$  PFU) reveals total necrosis of the tumor with immunostaining found in the entire surgical pocket with multiple layers expressing protein, including stroma and superficial muscular layers. Panel D (Ad5CMV-*p53* at  $10^9$  PFU) shows similar results to that of Panel C, however increased exogenous *p53* expression throughout the surgical site and edema are prominent.

Using animals, which served as their own internal controls, implants of  $4.0 \times 10^6$  or more cells significantly increased the establishment of s.c. implants as compared to the tumor implantation of  $2.5 \times 10^6$  cells ( $P < 0.01$ ), even when treated at the surgical site with Ad5CMV-*p53* 48 h after inoculation. Allowing implanted cells to establish for 72 or 96 h prior to the Ad5CMV-*p53* intervention similarly increased tumor take (data not shown). Dose-response experiments established that  $10^8$  and  $10^9$  PFUs of the Ad5CMV-*p53* were equally effective in inhibiting tumor burdens of  $2.5 \times 10^6$  cells implanted for 48 h (data not shown). Endogenous *p53* status of implanted tumor cell lines (whether homozygous mutated or wild-type *p53*) had little impact on the effectiveness of the Ad5CMV-*p53* in the cessation of tumor development.

Table 1 Effect of Ad5CMV-*p53* on tumorigenicity in a microscopic residual disease model of SCCN

Forty-eight h post tumor cell implantation, mice were again anesthetized and given a single intervention of either vehicle or viruses [ $1 \times 10^8$  PFU (Ad5CMV-*p53* or d1312) each in 0.1 ml] in each of the flap sites. Tumor formation was evaluated at the end of a 12-week period. In further experiments, the wild-type *p53* cell line, MDA 886, exhibited no tumor developed in 5 of 5 animals when the identical treatment strategy with  $10^8$  PFU Ad5CMV-*p53* was delivered at 48 h.

Cell line	Treatment		
	No. of mice developing tumors/total mice		
	PBS	d1312	Ad5CMV- <i>p53</i>
Tu-138 (homozygous mutation <i>p53</i> )	8/8	8/8	0/8
Tu-177 (homozygous mutation <i>p53</i> )	8/8	8/8	0/8
686-LN (homozygous wild-type <i>p53</i> )	5/8	5/8	0/8
886 (homozygous wild-type <i>p53</i> )	6/6	6/6	2/6

## Discussion

Tumor suppressor genes are only one of several groups of genes whose transfer might be useful for the local and regional treatment of cancer. Increasing immune surveillance by enhancing of MHC antigen expression or inducing local cytokines to activate the immune system can induce a local tumor effect. Another approach is introducing a gene that may induce apoptosis or that would make a tumor sensitive to particular chemotherapeutic agents. Furthermore, focusing on cell-cycle arrest in malignancies may prove beneficial.

At least two gene "suicide vectors" have already been described; the herpes simplex thymidine kinase gene allows infected cells to be treated with ganciclovir while noninfected cells are unaffected, and the bacterial enzyme cytosine deaminase gene allows infected cells to convert 6'-fluorocytosine to 5-fluorouracil. Unlike these agents, Ad5CMV-*p53* has shown no significant toxic effect on untransformed cells and its expression is transient, lasting about 15 days (data not shown).

*In vitro* experiments have shown that Ad5CMV-*p53* stops cell growth regardless of the endogenous *p53* gene status of the tumor cells. The mechanism by which abundant overexpression of wild-type *p53* protein induces this effect appears to be apoptosis, but this requires further investigation. It is important that normal fibroblasts transduced by this vector express the wild-type protein at levels similar to the SCCN cell lines but without inhibition of cell growth or abnormalities in morphology. The unique molecular events occurring within these transformed tumor cell lines that dispose them to cell death following Ad5CMV-*p53* transduction (while nonmalignant cells are spared) requires elucidation. Nevertheless, this sparing of normal cells further supports the potential for molecular therapy with this vector, since transduction of normal cells will be unavoidable in *in vivo* human trials.

Clearly a pure viral effect was also noted on delivery to the s.c. pockets, however this did not appear to be tumoricidal in these experiments. No clinical or histological findings of inflammation or edema different from those at the control sites were noted when the mice were treated with replication-defective virus or adenovirus  $\beta$ -galactosidase at  $10^7$  PFU or less. At  $10^8$  PFU and higher doses, however, inflammatory polymorphonuclear leukocytes and edema were histopathologically evident in the pocket sites, although no soft-tissue compromise was clinically evident. These conditions were not seen in mice mock-infected with transport medium alone or with lower viral doses.

*In vitro* studies in our laboratory have shown approximately 70% adenovirus transduction among SCCN cell lines in a single exposure under optimal conditions. Nevertheless, the *in vivo* studies clearly showed significant suppression of tumor development from tumor burdens of  $2.5 \times 10^6$  cells. Whether this reflects a need for a particular minimum tumor burden for tumors to develop, a "bystander" infectious environment, or an insensitivity of our assays to determine the effectiveness of molecular transfer has not been established.

These studies were designed to evaluate the efficacy and potential therapeutic role of *in vivo* gene transfer in a local-regional microscopic residual disease model. They revealed an effective *in vivo* mechanism for molecular therapy in head and neck cancer and suggest that this model delivery system may have a profound impact in the management of microscopic residual disease.

## References

- American Cancer Society Facts and Figures. Publ. No. 93-400. Washington, DC: American Cancer Society, 1993.
- Merino, O. R., Lindberg, R. D., and Fletcher, G. H. An analysis of distant metastasis from squamous cell carcinoma of the upper respiratory and digestive tracts. *Cancer (Phila.)*, 40: 147-156, 1977.

3. Thawley, S. E., and Panje, W. R. (eds.). *Comprehensive Management of Head and Neck Tumors*, Vol. 2, pp. 1158-1172. Philadelphia: W. B. Saunders Co., 1987.
4. Kozarsky, K. F., and Wilson, J. M. Gene therapy: adenovirus vectors. *Curr. Opin. Genet. Dev.*, 3: 499-503, 1993.
5. Mulligan, R. C. The basic science of gene therapy. *Science* (Washington DC), 260: 926-932, 1993.
6. Clayman, G., Wang, S. W., Nicolson, G. L., El-Naggar, A., Mazar, A., Henkins, J., Blasi, F., Goepfert, H., and Boyd, D. D. Regulation of urokinase-type plasminogen activator expression in squamous-cell carcinoma of the oral cavity. *Int. J. Cancer*, 54: 73-80, 1993.
7. Sacks, P. G., Pamea, S. M., Gallick, G. E., Mansouri, Z., Lichter, R., Satya-Prakash, K. L., Pathak, S., and Parsons, D. F. Establishment and characterization of two new squamous cell carcinoma cell lines derived from tumors of the head and neck. *Cancer Res.*, 48: 2858-2866, 1988.
8. Liu, T. J., Zhang, W. W., Taylor, D. L., Roth, J. A., Goepfert, H., Clayman, G. L. Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus. *Cancer Res.*, 54: 3662-3667, 1994.
9. Hsu, S. M., Raine, L., and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.*, 29: 577-580, 1981.



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**United States Patent** [19]

Roth et al.

[11] Patent Number: **5,747,469**[45] Date of Patent: **May 5, 1998****[54] METHODS AND COMPOSITIONS  
COMPRISING DNA DAMAGING AGENTS  
AND P53**

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..... **C12N 15/00**

[52] U.S. Cl. .... **514/44**; **435/172.3**; **435/240.1**;  
..... **435/240.2**; **435/240.21**; **435/320.1**; **514/2**

[58] Field of Search ..... **514/44**; **435/91**;  
..... **435/93.21**; **172.1**; **172.3**; **240.1**; **240.2**; **320.1**;  
..... **91.31**; **424/93.2**; **93.21**

**[56] References Cited****U.S. PATENT DOCUMENTS**

4,740,463	4/1988	Weinberg et al.	435/172.3
4,980,289	12/1990	Temin et al.	435/235.1
5,055,400	10/1991	Lo et al.	435/69.1
5,166,320	11/1992	Wu et al.	530/395
5,252,479	10/1993	Strivastava	435/235.1
5,328,470	7/1994	Nabel et al.	60/101
5,532,220	7/1996	Lee et al.	514/44

**FOREIGN PATENT DOCUMENTS**

0174608	9/1985	European Pat. Off.
0351585	6/1989	European Pat. Off.
0 390 323	10/1990	European Pat. Off.
0475623	8/1991	European Pat. Off.
2688514	9/1993	France
WO 90/10448	9/1990	WIPO
WO 93/03769	3/1993	WIPO
9408026	4/1994	WIPO
WO 94/10323	5/1994	WIPO
WO 94/24297	10/1994	WIPO
WO 95/02697	1/1995	WIPO

**OTHER PUBLICATIONS**

Baker, Suzanne J., et al., "Suppression of Human Colorectal  
Carcinoma Cell Growth by Wild-Type p53", *Science*, vol.  
249, pp. 912-915, Aug. 24, 1990.  
Bargonetti, Jill, et al., "Wild-Type but Not Mutant p53  
Immunopurified Proteins Bind to Sequences Adjacent to the  
SV40 Origin of Replication", *Cell*, vol. 65, pp. 1083-1091,  
Jun. 14, 1991.

Berkner, Kathleen L., "Development of Adenovirus Vectors  
for the Expression of Heterologous Genes", *BioTechniques*,  
vol. 6, No. 7, pp. 616-629, 1988.

Blenis, John, "Signal transduction via the MAP kinases:  
Proceed at your own RSK", *Proc. Natl. Acad. Sci. USA*, vol.  
90, pp. 5889-5892, Jul. 1993.

Brachman, David G., et al., "p53 Mutation Does Not Cor-  
relate with Radiosensitivity in 24 Head and Neck Cancer  
Cell Lines", *Cancer Research*, vol. 53, pp. 3667-3669, Aug.  
15, 1993.

Capecchi, Mario R., "Altering the Genome by Homologous  
Recombination", *Science*, vol. 244, pp. 1288-1292, Jun. 16,  
1989.

Cheng, Jian, et al., "Suppression of Acute Lymphoblastic  
Leukemia by the Human Wild-Type p53 Gene", *Cancer  
Research*, vol. 52, pp. 222-226, Jan. 1, 1992.

Coleman, C. Norman and Turrissi, Andrew T., "Radiation and  
Chemotherapy Sensitizers and Protectors", *Critical Reviews  
In Oncology/Hematology*, vol. 10, Issue 3, pp. 225-252,  
1990.

Comings, David E., "A General Theory of Carcinogenesis",  
*Proc. Natl. Acad. Sci. USA*, vol. 70, No. 12, Part I, pp.  
3324-3328, Dec. 1973.

Friedman, M.D., Theodore, "Gene Therapy of Cancer  
through Restoration of Tumor-Suppressor Functions?",  
*Cancer Supplement*, vol. 70, No. 6, pp. 1810-1817, Sep. 15,  
1992.

Gudkov, Andrei V., et al., "Isolation of genetic suppressor  
elements, inducing resistance to topoisomerase II-interac-  
tion cytotoxic drugs, from human topoisomerase II cDNA",  
*Proc. Natl. Acad. Sci. USA*, vol. 90, pp. 3231-3235, Apr.  
1993.

Houghten, Richard A., "Peptide libraries: criteria and  
trends", *Technical Focus*, vol. 9, No. 7, pp. 235-239, Jul.  
1993.

Itoh, Naoto, et al., "The Polypeptide Encoded by the cDNA  
for Human Cell Surface Antigen Fas Can Mediate Apopto-  
sis", *Cell*, vol. 66, pp. 233-243, Jul. 26, 1991.

(List continued on next page.)

**Primary Examiner**—Jacqueline M. Stone**Assistant Examiner**—Andrew Milne**Attorney, Agent, or Firm**—Arnold, White & Durkee**[57] ABSTRACT**

The present invention relates to the use of tumor suppressor  
genes in combination with a DNA damaging agent or factor  
for use in killing cells, and in particular cancerous cells. A  
tumor suppressor gene, p53, was delivered via a recombi-  
nant adenovirus-mediated gene transfer both in vitro and in  
vivo, in combination with a chemotherapeutic agent. Treated  
cells underwent apoptosis with specific DNA fragmentation.  
Direct injection of the p53-adenovirus construct into tumors  
subcutaneously, followed by intraperitoneal administration  
of a DNA damaging agent, cisplatin, induced massive apo-  
ptotic destruction of the tumors. The invention also provides  
for the clinical application of a regimen combining gene  
replacement using replication-deficient wild-type p53 aden-  
ovirus and DNA-damaging drugs for treatment of human  
cancer.

## OTHER PUBLICATIONS

- Jayawickreme, Channa K., et al., "Creation and functional screening of a multi-use peptide library", *Proc. Natl. Acad. Sci. USA*, vol. 91, pp. 1614-1618, Mar. 1994.
- Kamb, Alexander, et al., "A Cell Cycle Regulator Potentially Involved in Genesis of Many Tumor Types", *Science*, vol. 264, pp. 436-440, Apr. 15, 1994.
- Kern, Scott E., et al., "Identification of p53 as a Sequence-Specific DNA-Binding Protein", *Science*, vol. 252, pp. 1708-1711, Jun. 21, 1991.
- Knudson, Jr., Alfred G., "Mutation and Cancer: Statistical Study of Retinoblastoma", *Proc. Natl. Acad. Sci. USA*, vol. 68, No. 4, pp. 820-823, Apr. 1971.
- Kuerbitz, Steven J., et al., "Wild-type p53 is a cell cycle checkpoint determinant following irradiation", *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 7491-7495, Aug. 1992.
- Li, Frederick P., et al., "A Cancer Family Syndrome in Twenty-four Kindreds", *Cancer Research*, vol. 48, pp. 5358-5362, Sep. 15, 1988.
- Malkin, David, et al., "Germ Line p 53 Mutations in a Familial Syndrome of Breast Cancer, Sarcomas, and Other Neoplasms", *Science*, vol. 250, pp. 1233-1238, Nov. 30, 1990.
- Mansour, Suzanne L., et al., "Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination", *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 7688-7692, Oct. 1990.
- Marshall, C. J., "Hot lips and phosphorylation of protein kinases", *Nature*, vol. 367, p. 686, Feb. 24, 1994.
- Mercer, W. Edward, et al., "Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53", *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 6166-6170, Aug. 1990.
- Michalovitz, Dan, et al., "Conditional Inhibition of Transformation and of Cell Proliferation by a Temperature-Sensitive Mutant of p53", *Cell*, vol. 62, pp. 671-680, Aug. 24, 1990.
- Nigro, Janice M., et al., "Mutations in the p53 gene occur in diverse human tumour types", *Nature*, vol. 342, pp. 705-708, Dec. 7, 1989.
- O'Connor, Patrick M., et al., "Role of the p53 Tumor Suppressor Gene in Cell Cycle Arrest and Radiosensitivity of Burkitt's Lymphoma Cell Lines", *Cancer Research*, vol. 53, pp. 4776-4780, Oct. 15, 1993.
- Paull, Kenneth D., et al., "The Synthesis of XTT: A New Tetrazolium Reagent that is Bio reducible to a Water-Soluble Formazan", *J. Heterocyclic Chem.*, vol. 25, pp. 911-914, May-Jun. 1988.
- Petty, Russell D., et al., "Expression of the p53 Tumour Suppressor Gene Product is a Determinant of Chemosen-sitivity", *Biochem. and Biophys. Research Communications*, vol. 199, No. 1, pp. 264-270, Feb. 28, 1994.
- Phillips, Margaret A., et al., "Transition-State Characterization: A New Approach Combining Inhibitor Analogues and Variation in Enzyme Structure", *Biochemistry*, vol. 31, pp. 959-963, 1992.
- Revet, Bernard M. J., et al., "Homologous DNA Targeting with RecA Protein-coated Short DNA Probes and Electron Microscope Mapping on Linear Duplex Molecules", *J. Mol. Biol.*, vol. 232, pp. 779-791, 1993.
- Saris, M.D., Stephen C., et al., "Treatment of murine primary brain tumors with systemic interleukin-2 and tumor-infiltrating lymphocytes", *J. Neurosurg.*, vol. 76, pp. 513-519, 1992.
- Shaulsky, Gad, et al., "Involvement of wild-type p53 in pre-B-cell differentiation in vitro", *Proc. Natl. Acad. Sci. USA*, vol. 88, pp. 8982-8986, Oct., 1991.
- Shay, Jerry W., et al., "A Role for Both RB and p53 in the Regulation of Human Cellular Senescence", *Experimental Cell Research*, vol. 196, pp. 33-39, 1991.
- Muzyczka, N., "Use of Adeno-Associated Virus as a General Transduction Vector for Mammalian Cells", *Current Topics in Microbiol. and Immunol.*, vol. 158, pp. 98-129, 1992.
- Srivastava, Shiv., et al., "Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome", *Nature*, vol. 348, pp. 747-749, Dec. 1990.
- Takahashi, Takashi, et al., "p53: A Frequent Target for Genetic Abnormalities in Lung Cancer", *Science*, vol. 246, pp. 491-494, Oct. 27, 1989.
- Thomas, Kirk R., et al., "High-Fidelity Gene Targeting in Embryonic Stem Cells by Using Sequence Replacement Vectors", *Molecular and Cellular Biol.*, vol. 12, No. 7, pp. 2919-2923, Jul. 1992.
- Ullrich, Stephen J., et al., "Human wild-type p53 adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells", *Oncogene*, vol. 7, pp. 1635-1643, 1992.
- Unger, Tamar, et al., "Functional Domains of Wild-Type and Mutant p53 Proteins Involved in Transcriptional Regulation, Transdominant Inhibition, and Transformation Suppression", *Molecular and Cellular Biol.*, vol. 13, No. 9, pp. 5186-5194, Sep., 1993.
- Vogelstein, Bert and Kinzler, Kenneth W., "p53 Function and Dysfunction", *Cell*, vol. 70, pp. 523-526, Aug. 21, 1992.
- Wagner, Ernst, et al., "Coupling of adenovirus to transferin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes", *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 6099-6103, Jul. 1992.
- Weislow, Owen S., et al., "New Soluble-Formazan Assay for HIV-1 Cytopathic Effects: Application to High-Flux Screening of Synthetic and Natural Products for AIDS-Antiviral Activity", *J. Natl. Cancer Inst.*, vol. 81, No. 8, pp. 577-586, Apr. 19, 1989.
- Wu, George Y., et al., "Receptor-mediated Gene Delivery in vivo", *The J. of Biol. Chem.*, vol. 266, No. 22, pp. 14338-14342, Aug. 5, 1991.
- Yonish-Rouach, Elisheva, et al., "Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6", *Nature*, vol. 352, pp. 345-347, Jul. 25, 1991.
- Yoshimura, Kunihiko, et al., "Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after in vivo intratracheal plasmid-mediated gene transfer", *Nucleic Acid Research*, vol. 20, No. 12, pp. 3233-3240, Apr. 1992.
- Zhu, Ning, et al., "Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice", *Science*, vol. 261, pp. 209-211, Jul. 9, 1993.
- Singleton, Paul and Sainsbury, Diana, *Dictionary of Microbiology and Molecular Biology*, 2nd Ed., John Wiley & Sons, Publ., pp. 591 and 920.
- Stedman's Medical Dictionary*, 25th Ed., Williams & Wilkins, Publ., p. 245.
- Comprehensive Textbook of Oncology*, vol. 1, 2nd Ed., Williams & Wilkins, Publ., pp. 477, 527-536, 565-568, 590-594, 607-612.

- Comprehensive Textbook of Oncology*, vol. 2, 2nd Ed., Williams & Wilkins, Publ., pp. 1098, 1138-1140, 1170, 1329, 1368, 1569-1572.
- Federal Register*, vol. 47, No. 56, pp. Title -VI and i-iv, Mar. 23, 1982.
- Steel, Michael, "Cyclins and cancer: wheels within wheels", *The Lancet*, vol. 343, pp. 931-932, Apr. 16, 1994.
- Proceedings of the American Association for Cancer Research*, vol. 36, p. 21, Mar. 1995.
- Fujiwara, Toshiyoshi, et al., "Induction of Chemosensitivity in Human Lung Cancer Cells in vivo by Adenovirus-mediated Transfer of the Wild-Type p53 Gene", *Cancer Research*, vol. 54, pp. 2287-2291, May 1, 1994.
- Diller, Lisa, et al., "p53 Functions as a Cell Cycle Control Protein in Osteosarcomas", *Molecular and Cellular Biology*, vol. 10, No. 11, pp. 5772-5781, Nov. 1990.
- Cureil, et al., 3, "High-efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes", *Human Gene Therapy*, 147-154 (1992).
- Foreman, et al., 4(3) Title Unknown, *Bone Marrow Transplantation* (1990).
- Graham, et al., 7 Title Unknown, *Methods in Molecular Biology, Gene Transfer and Expression Protocols*, Ch. 11, pp. 109-128 (1991).
- Hinds, et al., *Cell Growth and Differentiation*, 571-580 (1990).
- Huston, et al., 10(2-3) "Medical applications of single-chain antibodies", *International Reviews of Immunology*, 195-217 (1993).
- Jolly, D., 1(1) "Viral vector systems for gene therapy", *Cancer Gene Therapy*, 51-64 (1994).
- Kriegler, et al., *Gene Transfer and Expression: A Laboratory Manual*.
- Romer, K. and Friedman, T., 716 "Annals of the New York Academy of Science, Gene Therapy for Neoplastic Diseases" 265-282 (1994).
- Van de Waterbeemd, 9 "Recent progress in QSAR-technology", *Drug Design and Discovery* 277-285 (1993).
- Su et al., "Transformation and radiosensitivity of human diploid skin fibroblasts transfected with SV40 T-antigen mutants defective in RB and P53 binding domains," *Int. J. Radiat. Biol.*, 1992 vol. 62, No. 4, 461-468.
- Petty et al., "Expression of the p53 Tumour Suppressor Gene Product is a Determinant of Chemosensitivity," *Biochemical and Biophysical Research Communications*, 199(1):264-270, Feb. 1994.
- PCT Search Report dated Apr. 24, 1995.
- Brown et al., "Mutant p53 Confers Cisplatin-sensitivity to Resistant Ovarian Tumour Cells with Elevated Wild-Type p53," *Proc. Am. Assoc. Cancer Res.*, 34:355, #2116, 1993.
- Donehower, "Tumor Suppressor Gene p53 and Apoptosis," *Cancer Bulletin* 46(2):161-166, 1994.
- El Rouby et al., "p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression," *Blood*, 82(11):3452-3459, 1993.
- Fan et al., "The role of p53 in cell cycle arrest and apoptosis induced by multiple chemotherapeutic agents in Burkitt's lymphoma cells," *Proceedings of the American Association for Cancer Research*, 35:311, #1851, 1994.
- Fornace, Jr., "Induction by radiation of mammalian genes associated with growth-arrest and apoptosis, and the role of the p53 tumor suppressor in their regulation," *Proceedings of the American Association for Cancer Research*, 35:682-682, 1994.
- Fritsche et al., "Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents", published erratum, *Oncogene*, 8(9):2605, 1993.
- Hecht et al., "Comparison of wildtype and mutated p53 protein expression induced by UV irradiation of cultured cells," *FASEB Journal*, 8:A667, #3870, 1994.
- Kaneko et al., "Induction of apoptosis and p53 protein by adriamycin and hyperthermia in a rat mammary adenocarcinoma cell line," *Proceedings of the American Association for Cancer Research*, 35:314, #1871, 1994.
- Kastan et al., "Participation of p53 protein in the cellular response to DNA damage," *Cancer Res.*, 51:6304-11, 1991.
- Kastan, "p53: a determinant of the cell cycle response to DNA damage," *Adv. Exp. Med. Biol.*, 339:295-296, 1993.
- Kastan et al., "p53 and other molecular controls of the response to DNA damage," *Journal of Cellular Biochemistry*, 9(18C):164, 1994.
- Kemp et al., "p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis," *Nature Genetics*, 8(1):66-69, 1994.
- Lancet, "A death in the life of p53," *Nature*, 362:786-787, 1993.
- Lee & Bernstein, "p53 mutations increase resistance to ionizing radiation," *Proc. Natl. Acad. Sci. USA*, 90(12):5742-5746, 1993.
- Levine et al., "The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis," *Br. J. Cancer*, 69(3):409-416, 1994.
- Loganzo, Jr. et al., "Stabilization of p53 protein is a critical response to UV radiation in human melanocytes: Implications for melanoma development," *Molecular and Cellular Differentiation*, 2(1):23-43, 1994.
- Lotem & Sachs, "Regulation by bcl-2, c-myc and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and -defective myeloid leukemic cells," *Cell Growth Differ.*, 4(1):41-7, 1993.
- Lotem & Sachs, "Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents," *Blood*, 82(4):1092-6, 1993.
- Maity et al., "The molecular basis for cell cycle delays following ionizing radiation: a review," *Radiother. Oncol.*, 31(1):1-13, 1994.
- McIlwraith et al., "Cell cycle arrests and radiosensitivity of human tumor cell lines: Dependence on wild-type p53 for radiosensitivity," *Cancer Research*, 54(14):3718-3722, 1994.
- Nabeya et al., "The mutational status of p53 protein in gastric cancer cell lines predicts sensitivity to chemotherapeutic agents," *Proceedings of the American Association for Cancer Research*, 35:602, #3591, 1994.
- O'Connor et al., "Relationship between p53, cyclin E-cdk2 kinase complexes and G1 arrest induced by ionizing radiation in human cells," *Proceedings of the American Association for Cancer Research*, 35:635, #3785, 1994.
- Rau et al., "Response of p53 to treatment with actinomycin D in human mammary carcinoma cell lines," *Journal of Cancer Research and Clinical Oncology*, 120:R108, 1994.
- Shaw et al., "Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line," *Proc. Natl. Acad. Sci. USA*, 89(10):4495-4499, 1992.
- Slichenmyer et al., "Loss of a p53-associated G1 checkpoint does not decrease cell survival following DNA damage," *Cancer Res.*, 53(18):4164-4168, 1993.

- Varghese et al., "The role of p53 and ras genes in radiation-induced transformation of immortalized human epidermal keratinocytes," *Proceedings of the American Association for Cancer Research*, 35:91, #542, 1994.
- Yamada & Ohyama, "Radiation and apoptosis," *Gan To Kagaku Ryoho*, 21(5):602-607, 1994, Abstract only.
- Bandyopadhyay & Temin, "Expression of Complete Chicken Thymidine Kinase Gene Inserted in a Retrovirus Vector," *Molecular and Cellular Biology*, 4(4):749-754, 1984.
- Bowtell et al., "Comparison of Expression in Hemopoietic Cells by Retroviral Vectors Carrying Two Genes," *Journal of Virology*, 62(7):2464-2473, 1988.
- Casson et al., "p53 Gene Mutations in Barrett's Epithelium and Esophageal Cancer," *Cancer Research*, 51:4495-4499, 1991.
- Chen et al., "Genetic Mechanisms of Tumor Suppression by the Human p53 Gene," *Science*, 250:1576-1580, 1990.
- Chen et al., "Expression of Wild-Type p53 in Human A673 Cells Suppresses Tumorigenicity but Not Growth Rate," *Oncogene*, 6:1799-1805, 1991.
- Goyette et al., "Progression of Colorectal Cancer Is Associated with Multiple Tumor Suppressor Gene Defects but Inhibition of Tumorigenicity Is Accomplished by Correction of Any Single Defect via Chromosome Transfer," *Molecular and Cellular Biology*, 12(3):1387-1395, 1992.
- Gusterson et al., "Expression of p53 in Premalignant and Malignant Squamous Epithelium," *Oncogene*, 6:1785-1798, 1991.
- Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science*, 248:1101-1104, 1990.
- Maxwell et al., "Inefficiency of Expression of Luciferase Reporter from Transfected Murine Leukaemia Proviral DNA May Be Partially Overcome by Providing a Strong Polyadenylation Signal," *Journal of General Virology*, 72:1721-1724, 1991.
- Mukhopadhyay et al., "Specific Inhibition of K-ras Expression and Tumorigenicity of Lung Cancer Cells by Antisense RNA," *Cancer Research*, 51:1744-1748, 1991.
- Owens & Boyd, "Expressing Antisense Po RNA in Schwann Cells Perturbs Myelination," *Development*, 112:639-649, 1991.
- Palmer et al., "Efficient Retrovirus-Mediated Transfer and Expression of a Human Adenosine Deaminase Gene in Diploid Skin Fibroblasts from an Adenosine Deaminase-Deficient Human," *Proceedings of the National Academy of Science USA*, 84:1055-1059, 1987.
- Seyama et al., "In Vitro and In Vivo Regulation of Liver Epithelial Cells Carrying a Metallothionein-rasT24 Fusion Gene," *Molecular Carcinogenesis*, 1:89-95, 1988.
- Takahashi et al., "Wild-Type but Not Mutant p53 Suppresses the Growth of Human Lung Cancer Cells Bearing Multiple Genetic Lesions," *Cancer Research*, 52:2340-2343, 1992.
- Zhou & Duesberg, "myc Protooncogene Linked to Retroviral Promoter, but Not to Enhancer, Transforms Embryo Cells," *Proceedings of the National Academy of Science USA*, 85:2924-2928, 1988.
- Conroy, "New Gene Therapy Cleared for Use Against Lung Cancer," *Biotech Daily*, pp. 3-4, Sep. 18, 1992.
- Dialog Search Report dated Sep. 22, 1992.
- Sundaresan, V., et al., "Somatic Genetic Changes in Pre-Invasive Lesions in Bronchial Epithelium," *J. Pathol.*, 167(Suppl) 1992, 100A, Abstract Only.
- Dialog Search Reports dated Aug. 7, 1992 and Feb. 26, 1993.
- Debus et al., *J Cancer Res Clin Oncol*, 116 (Suppl Part 1):5-162, Abstract # A2.037.09, 1990.
- Delauney et al., "A stable bifunctional antisense transcript inhibiting gene expression in transgenic plants," *Proc. Natl. Acad. Sci. USA*, 85:4300-4304, 1988.
- Feig, et al., "Somatic Activation of ras<sup>K</sup> Gene in a Human Ovarian Carcinoma," *Science*, 223:698-701, 1984.
- Finkel, et al., "Activation of ras Genes in Human Tumors Does Not Affect Localization, Modification, or Nucleotide Binding Properties of p21," *Cell*, 37:151-158, 1984.
- Griep and Heiner, "Antisense Myc sequences induce differentiation of F9 cells," *Proc. Natl. Acad. Sci. USA*, 85:6806-6810, 1988.
- Gunning, et al., "A human  $\beta$ -actin expression vector system directs high-level accumulation of antisense transcripts," *Proc. Natl. Acad. Sci. USA*, 84:4831-4835, 1987.
- Kasid, et al., "Effect of Antisense c-ras-1 on Tumorigenicity and Radiation Sensitivity of a Human Squamous Carcinoma," *Science*, 243:1354-1356, 1989.
- Khokha, Rama, et al., "Antisense RNA-Induced Reduction in Murine TIMP Levels Confers Oncogenicity on Swiss 3T3 Cells," *Science*, 243:947-950, 1989.
- Kris, et al., "Expression of Ki-Ras Oncogene in Tumor Cell Variants Exhibiting Different Metastatic Capabilities," *Int. J. Cancer*, 35:227-230, 1985.
- Izant and Weintraub, "Inhibition of Thymidine Kinase Gene Expression by Anti-Sense RNA: A Molecular Approach to Genetic Analysis," *Cell*, 36:1007-1015, 1984.
- Johnson, et al., "Transfection of a Rat Cell Line with the v-Ki-ras Oncogene is Associated with Enhanced Susceptibility to Natural Killer Cell Lysis," *J. Exp. Med.*, 162:1732-1737, 1985.
- McGrath, et al., "Structure and organization of the human Ki-ras protooncogene and a related processed pseudogene," *Nature*, 304:501, 1983.
- Magrath, "Tumor-specific antisense oligonucleotides for controlling cancer", Abstract No. 114:55778n, *Chemical Abstracts*, 114(7):68 (1991).
- Mercola, et al., "Antisense RNA: Eukaryotic Controls," *Gene*, 72:253-265 (1988).
- Miller and Rosman, Improved Retroviral Vectors for Gene Transfer and Expression, *BioTechniques*, 7(9):980-990, 1989.
- Mumroe, Stephen H., "Antisense RNA inhibits splicing of pre-mRNA in vitro", *The EMBO Journal*, 7(8):2523-2532 (1988).
- Prochownik, et al., "c-myc Antisense Transcripts Accelerate Differentiation and Inhibit G<sub>1</sub> Progression in Murine Erythroleukemia Cells", *Molecular and Cellular Biology*, 8(9):3683-3695, 1988.
- Santos, et al., Malignant Activation of a K-ras Oncogene in Lung Carcinoma but Not in Normal Tissue of the Same Patient, *Science*, 223:661-664, 1984.
- Shimizu, et al., "Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1", *Nature*, 304:497-500, 1983.
- Stowers, et al., "Activation of the K-ras Protooncogene in Lung Tumors from Rats and Mice Chronically Exposed to Tetranitromethane", *Cancer Research*, 47:3212-3219, 1987.
- Taya, et al., "A novel combination of K-ras and myc amplification accompanied by point mutational activation of K-ras in a human lung cancer", *The EMBO Journal*, 3(12):2943-2946, 1984.

- Toftgard, et al., "Proto-oncogene expression during two-stage carcinogenesis in mouse skin", *Carcinogenesis*, 6(4):655-657, 1985.
- Vogelstein, et al., "Genetic Alterations During Colorectal-Tumor Development", *The New England Journal of Medicine*, 319(9):525-532, 1988.
- Wahran et al., *Tumour Biol.* 6:41-56, 1985.
- Winter and Perucho, "Oncogene Amplification during Tumorigenesis of Established Rat Fibroblasts Reversibly Transformed by Activated Human ras Oncogenes", *Molecular and Cellular Biology*, 6(7):2562-2570, 1986.
- International Search Report, mailed Aug. 20, 1992.
- Brown et al., "Increased Accumulation of p53 Protein in Cisplatin-Resistant Ovarian Cell Lines," *Int. J. Cancer*, 55:678-684, 1993.
- Clarke et al., "Thymocyte apoptosis induced by p53-dependent and independent pathways," *Nature*, 362:849-852, 1993.
- El-Deiry et al., "WAF1, a Potential Mediator of p53 Tumor Suppression," *Cell*, 75:817-825, 1993.
- Fritsche et al., "Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents," *Oncogene*, 8:307-318, 1993.
- Fujiwara et al., "A Retroviral Wild-type p53 Expression Vector Penetrates Human Lung Cancer Spheroids and Inhibits Growth by Inducing Apoptosis," *Cancer Research*, 53:4129-4133, 1993.
- Harper et al., "The p21 Cdk-Interacting Protein Cip1 Is a Potent Inhibitor of G1 Cyclin-Dependent Kinases," *Cell*, 75:805-816, 1993.
- Lowe et al., "p53-Dependent Apoptosis Modulates the Cytotoxicity of Anticancer Agents," *Cell*, 74:957-967, 1993.
- Lowe et al., "p53 is required for radiation-induced apoptosis in mouse thymocytes," *Nature*, 362:847-849, 1993.
- Merritt et al., "The role of p53 in Spontaneous and Radiation-induced Apoptosis in the Gastrointestinal Tract of Normal and p53-deficient Mice," *Cancer Research*, 54:614-617, 1994.
- Tishler et al., "Increases in Sequence Specific DNA Binding by p53 following Treatment with Chemotherapeutic and DNA Damaging Agents," *Cancer Research*, 53:2212-2216, 1993.
- Casey et al., "Growth Suppression of Human Breast Cancer Cells by the Introduction of a Wild-Type p53 Gene," *Oncogene*, 6:1791-1797, 1991.
- Wills and Menzel, "Adenovirus Vectors for Gene Therapy of Cancer," *Journal of Cellular Biochemistry*, p. 204, Abstract # S216, Mar.-Apr. 1993.
- Zhang et al., "Generation and Identification of Recombinant Adenovirus by Liposome-Mediated Transfection and PCR Analysis," *BioTechniques*, 15(5):868-872, 1993.
- PCT Search Report dated Jul. 5, 1995.
- Marshall, *Science*, vol. 269, 1995 pp. 1050-1055.
- Neve, *Trends Neurosci.* vol. 16(7), 1993, pp. 251-253.
- Bachetti et al. *Int. J. Oncol.* vol. 3(5), 1993, pp. 781-788.
- Tishler et al. *Cancer Res.*, vol. 53, 1993 pp. 2212-2216.
- Culver et al, *Science*, vol. 256, 1992 pp. 1550-1552.

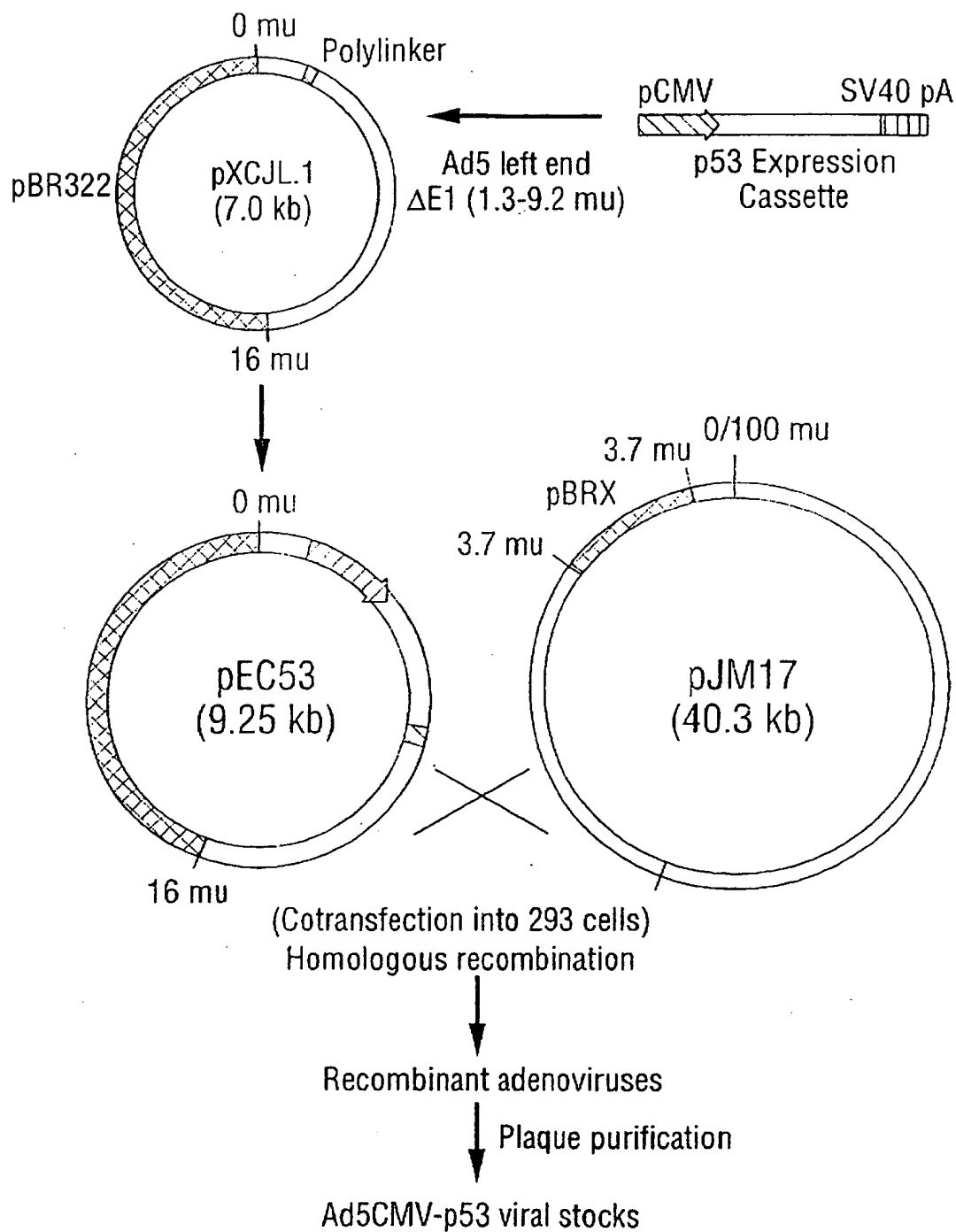


FIG. 1



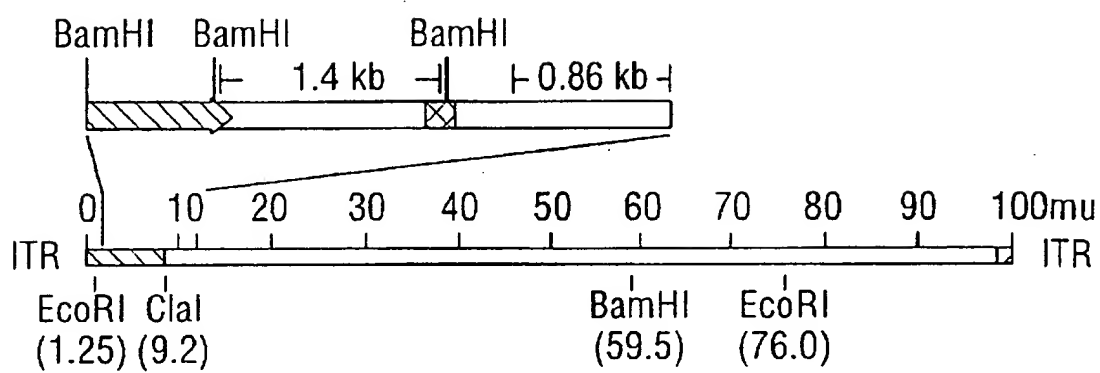


FIG. 2A

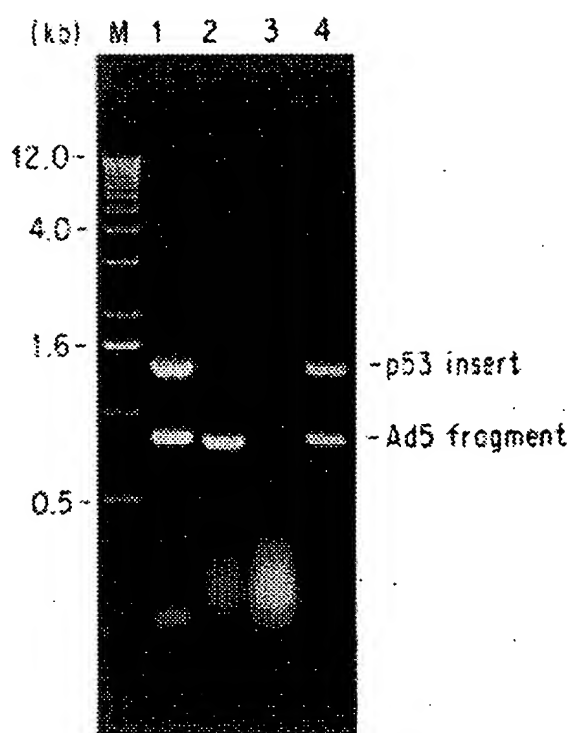


FIG.2B

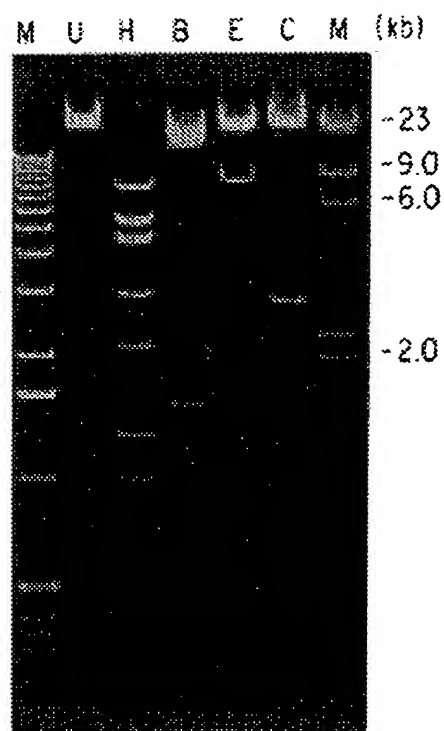


FIG.2C

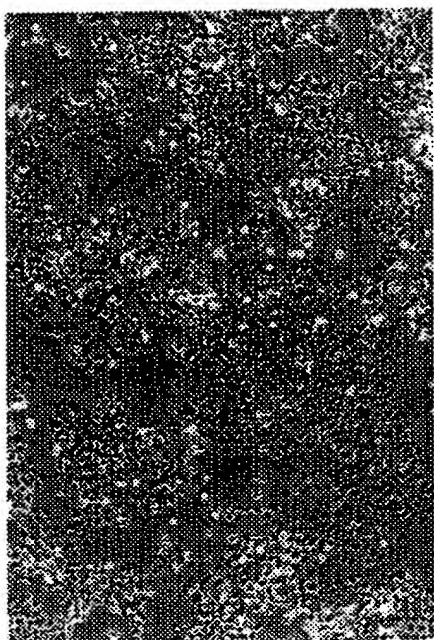


FIG. 3A

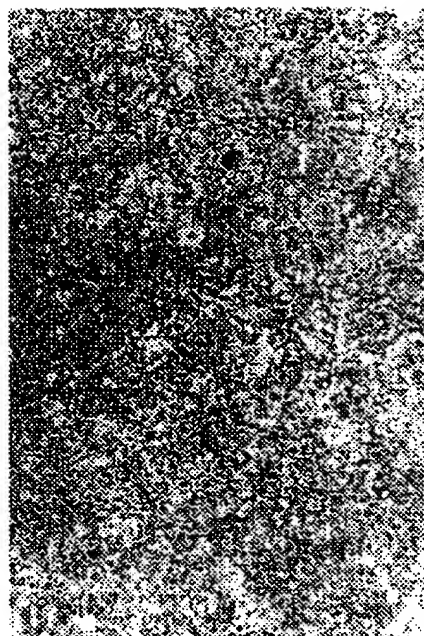


FIG. 3B

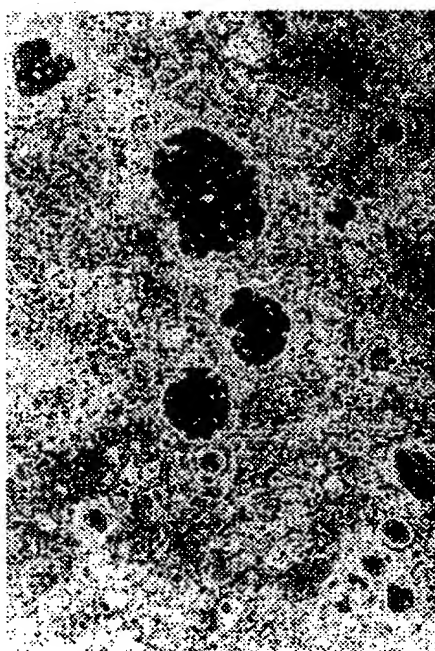


FIG. 3C

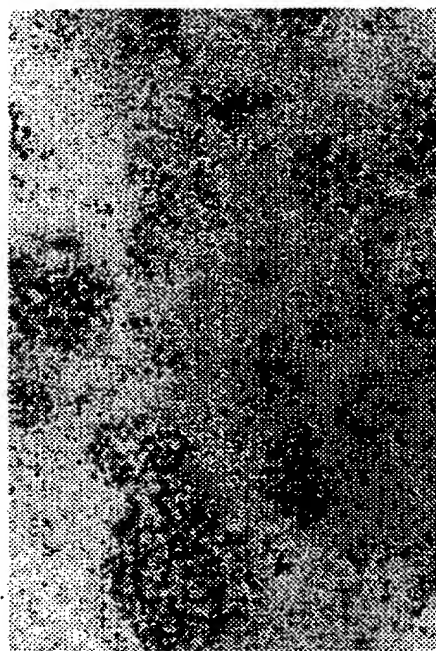


FIG. 3D

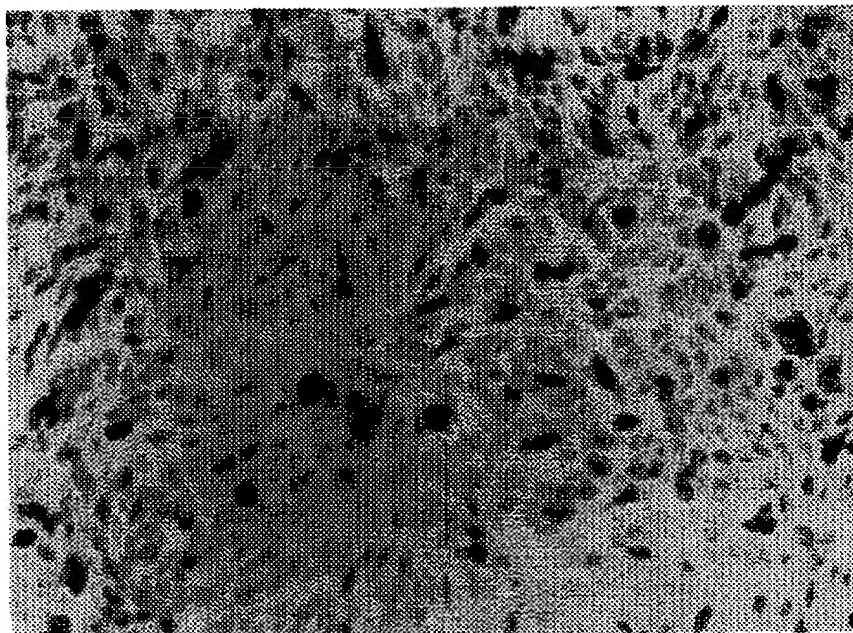


FIG.4A

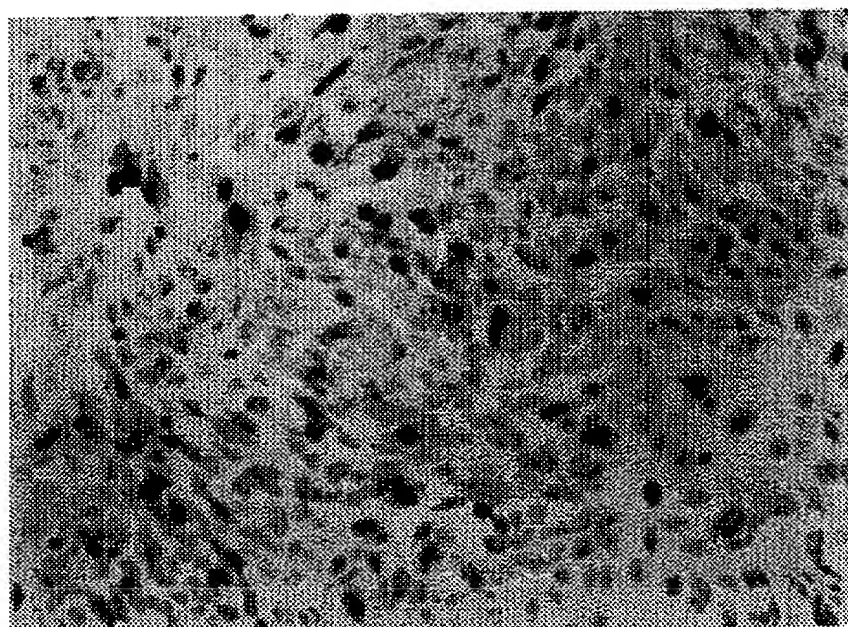


FIG.4B

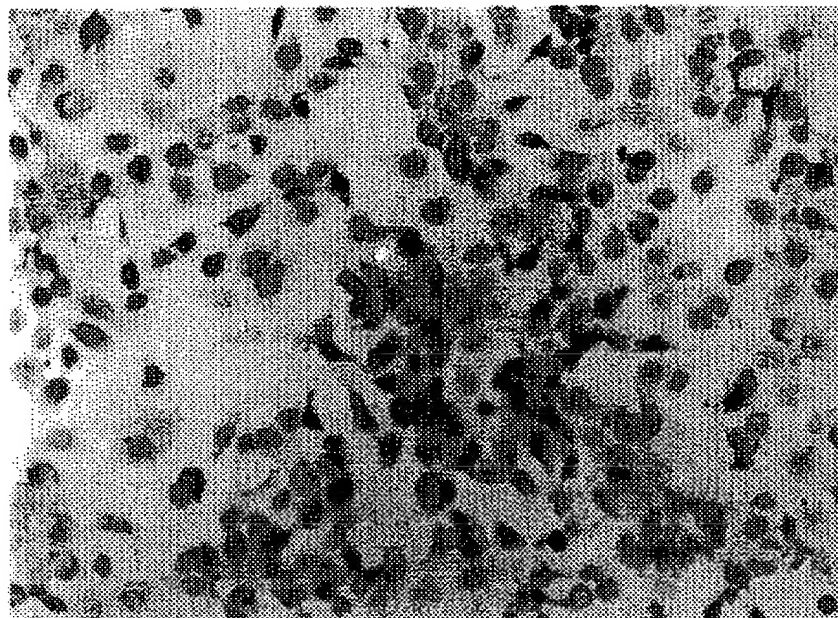


FIG.4C

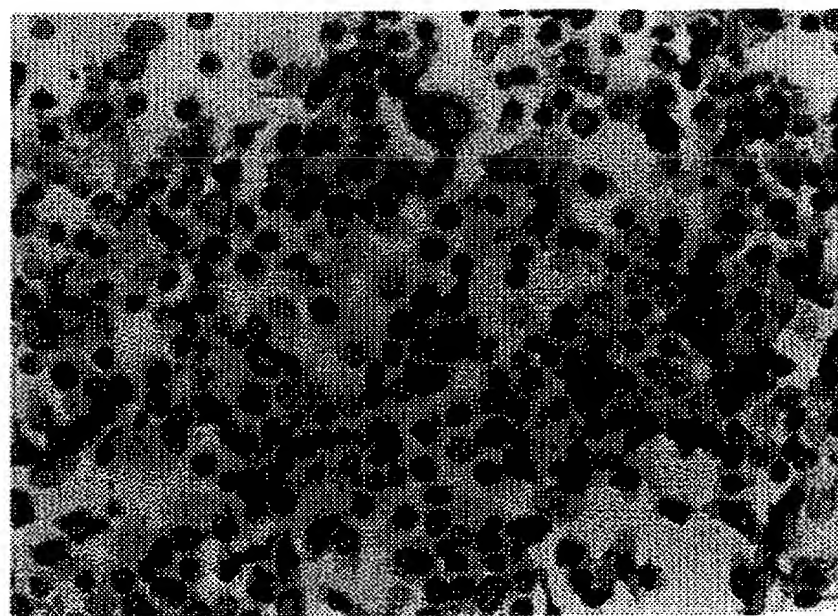


FIG.4D

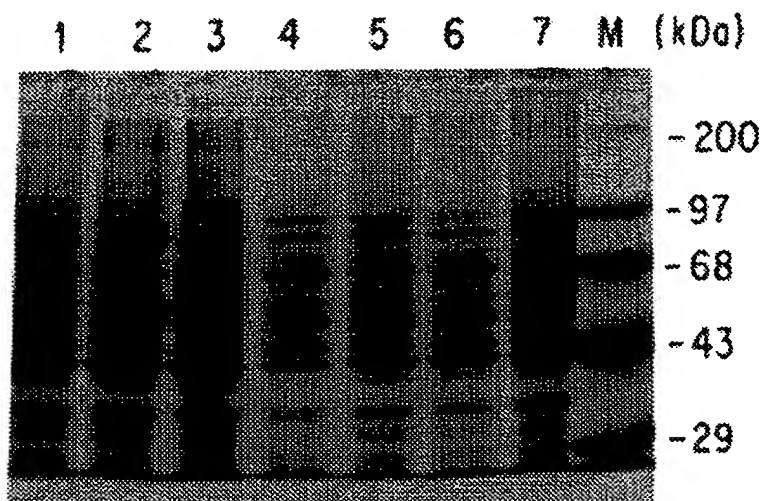


FIG.5A

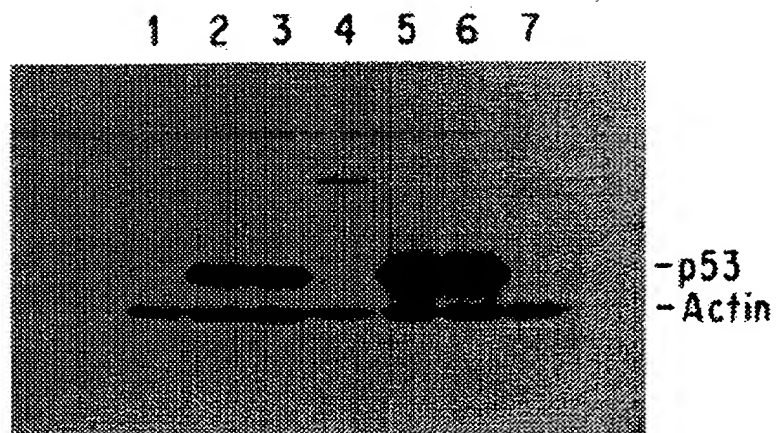


FIG.5B

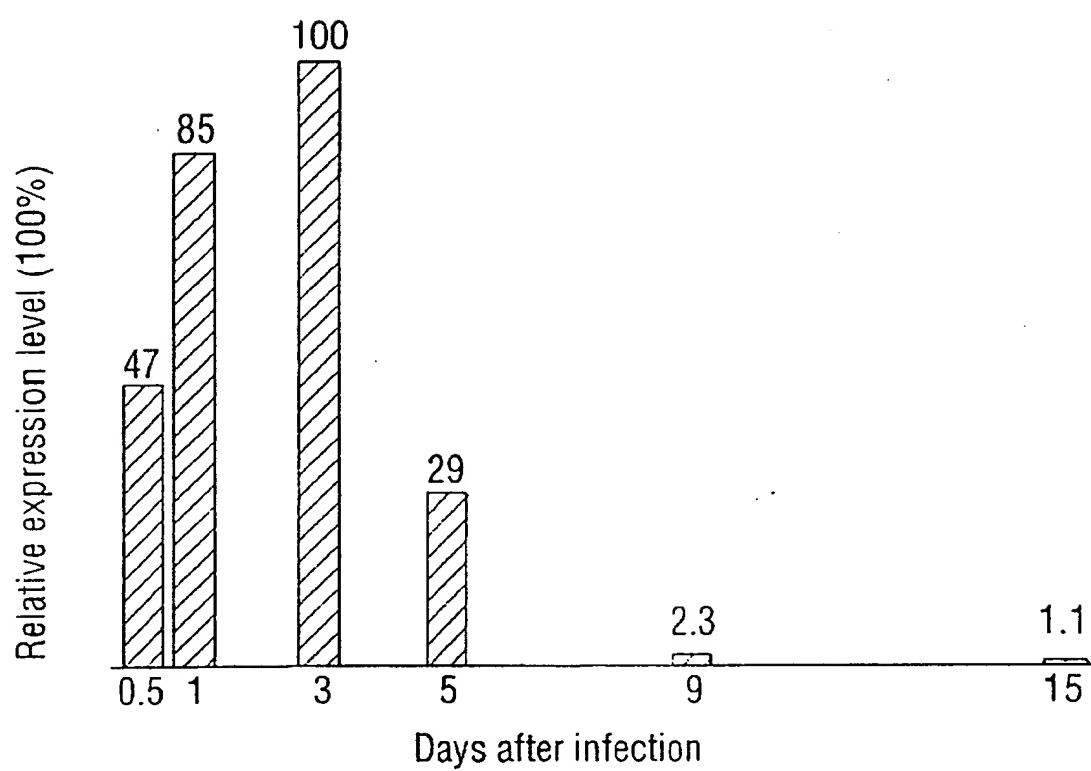


FIG. 6A



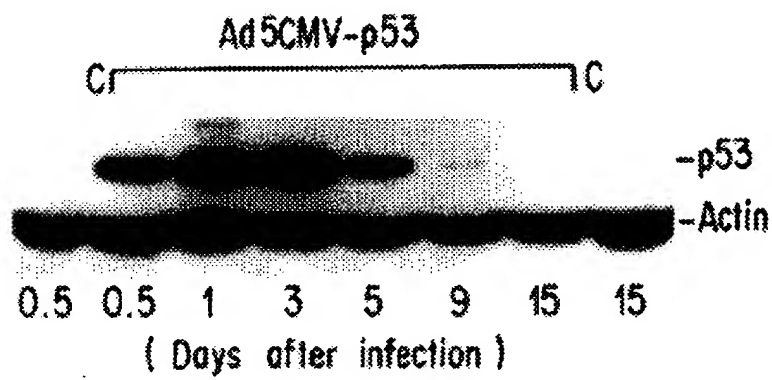


FIG.6B

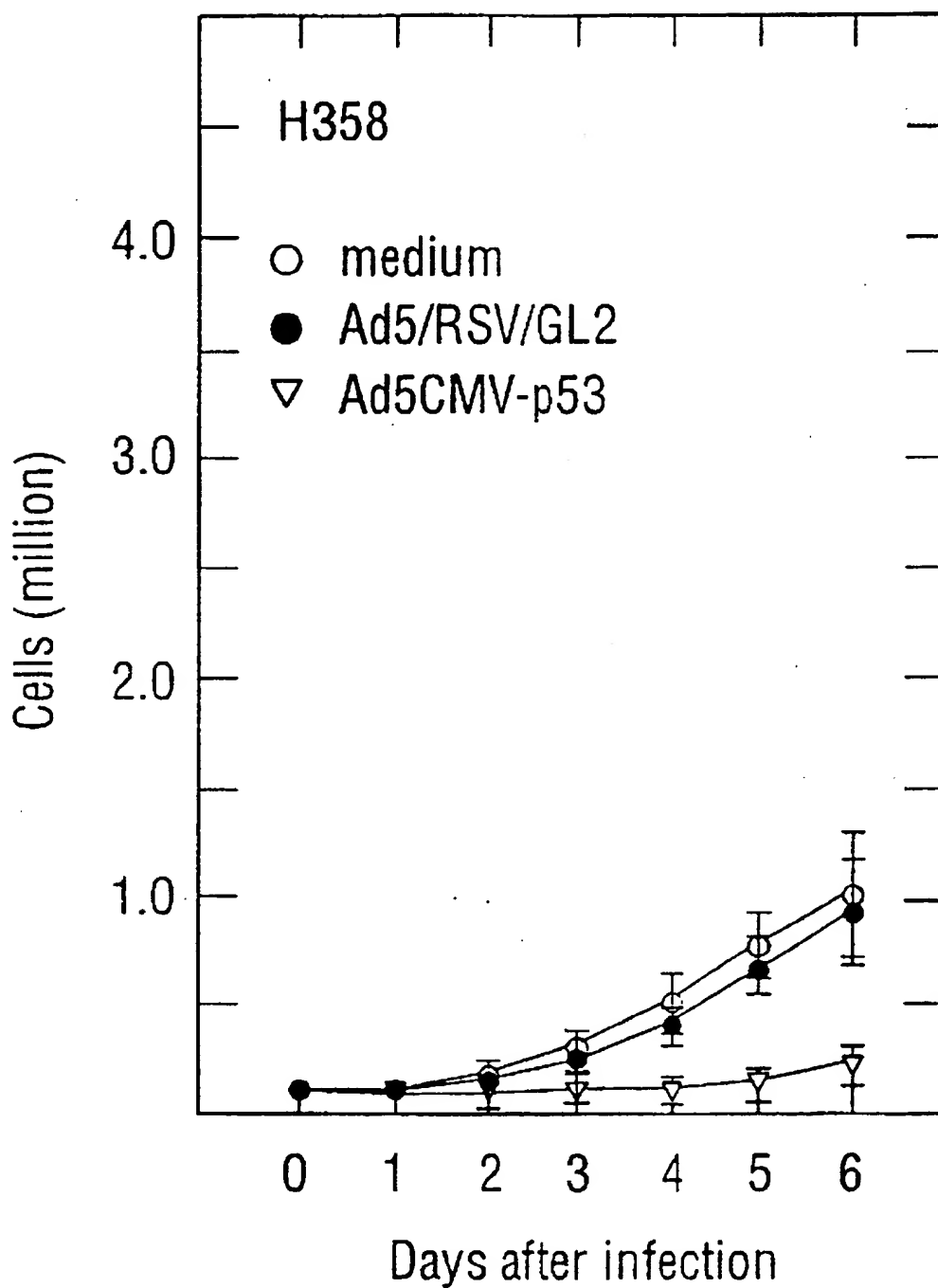


FIG. 7A

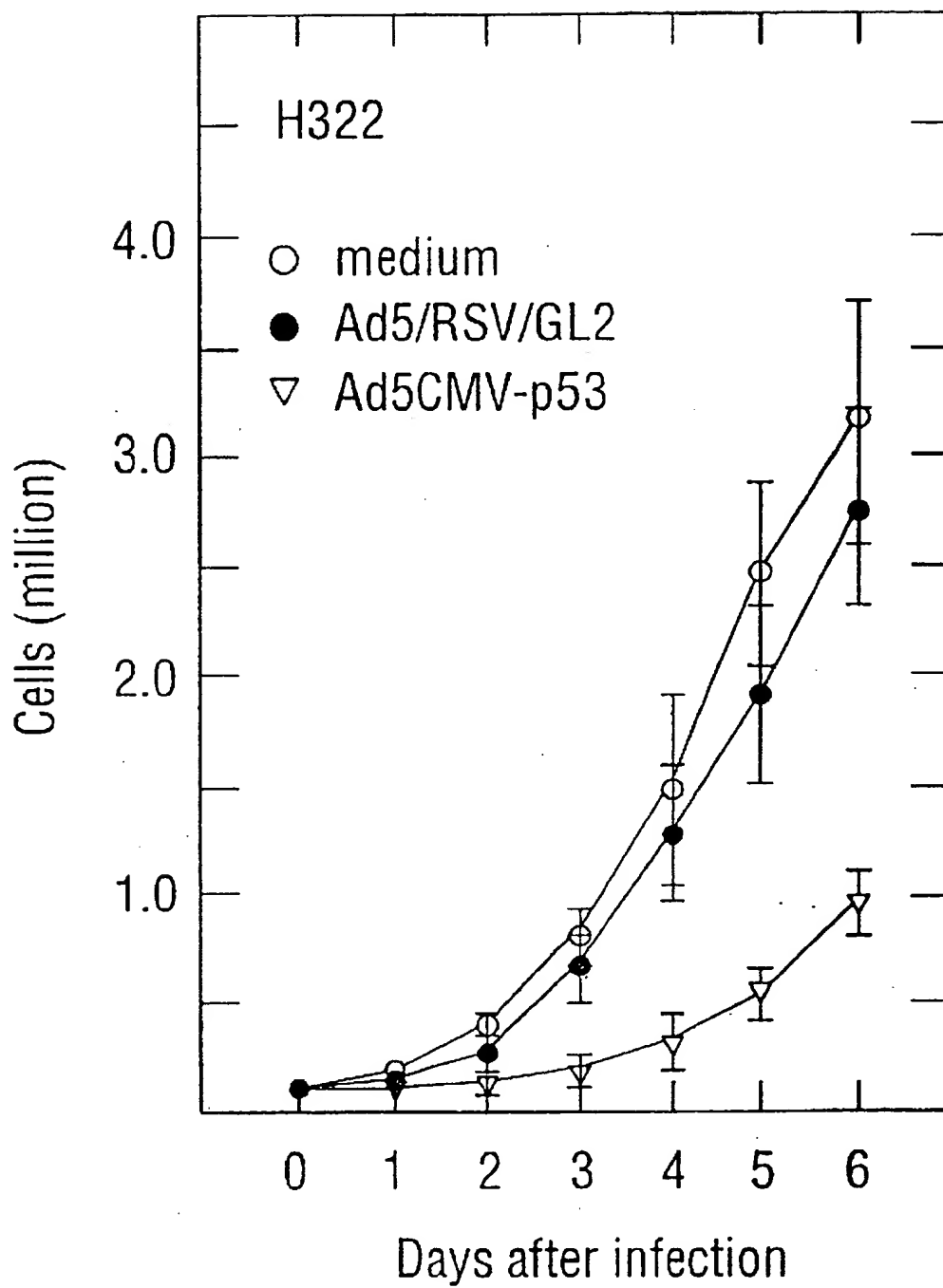


FIG. 7B

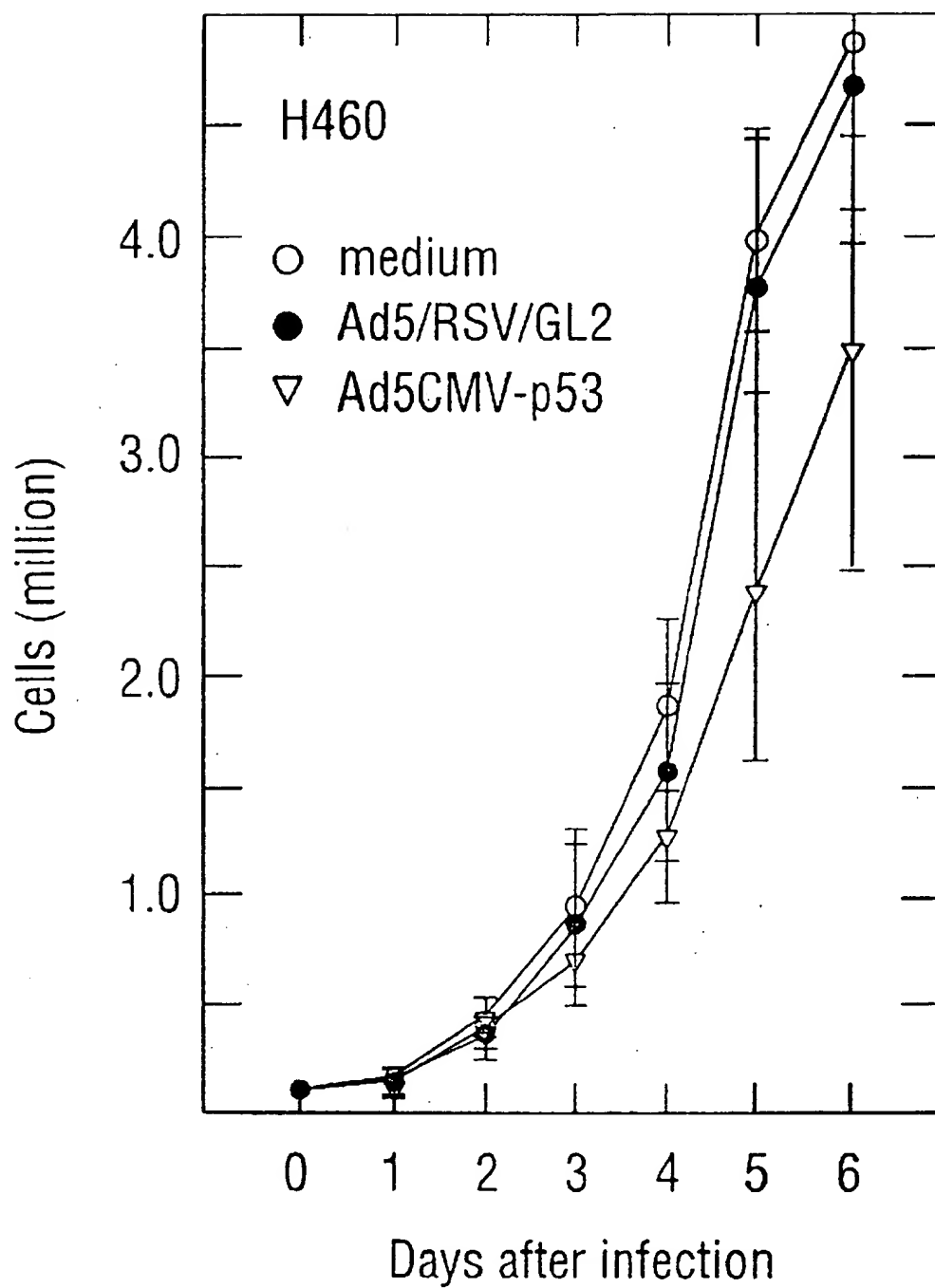


FIG. 7C

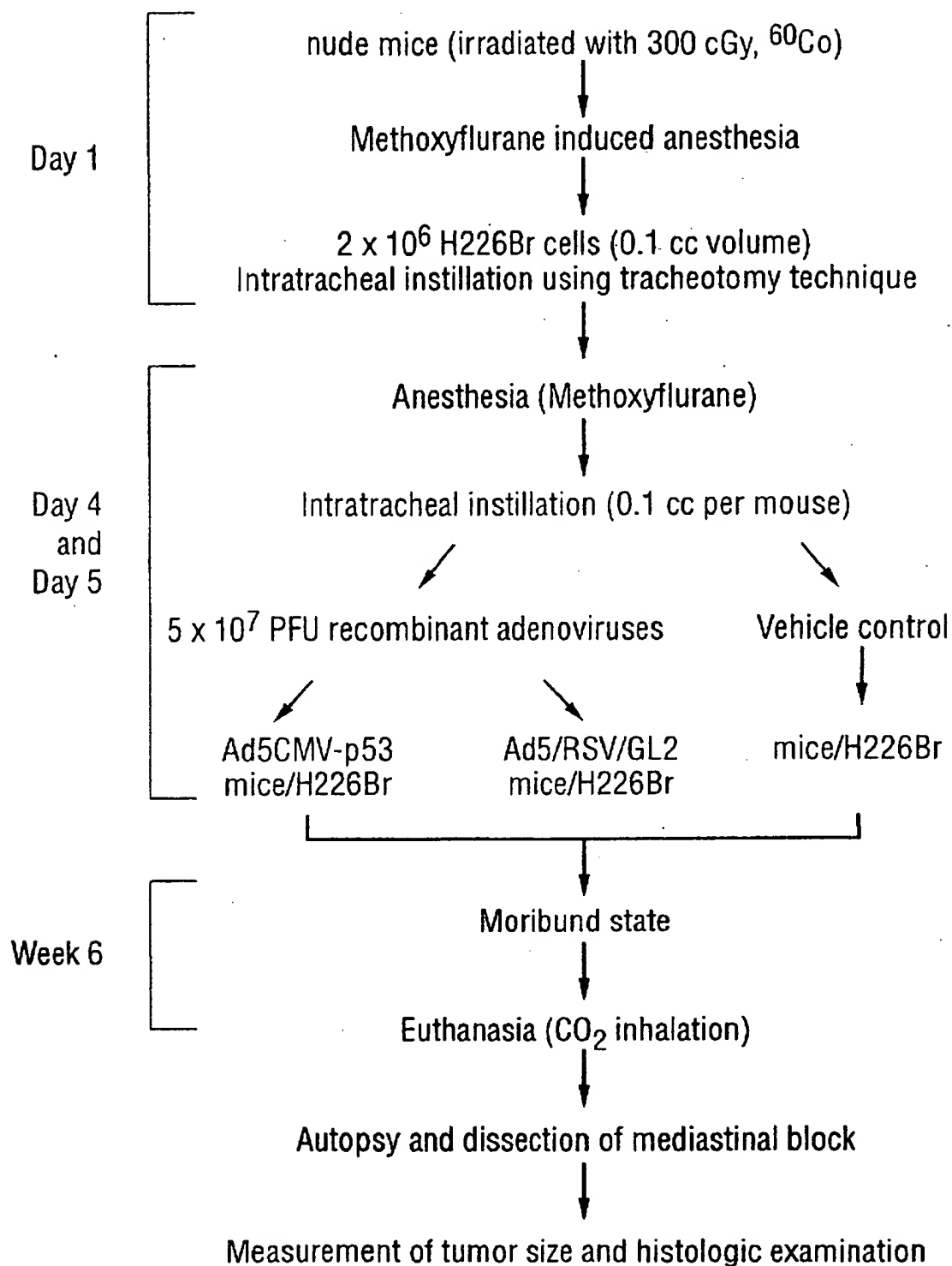


FIG. 8

FIG.9A

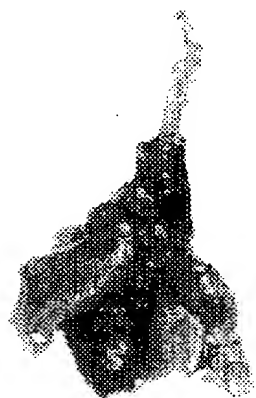


FIG.9B



FIG.9C



FIG.9D



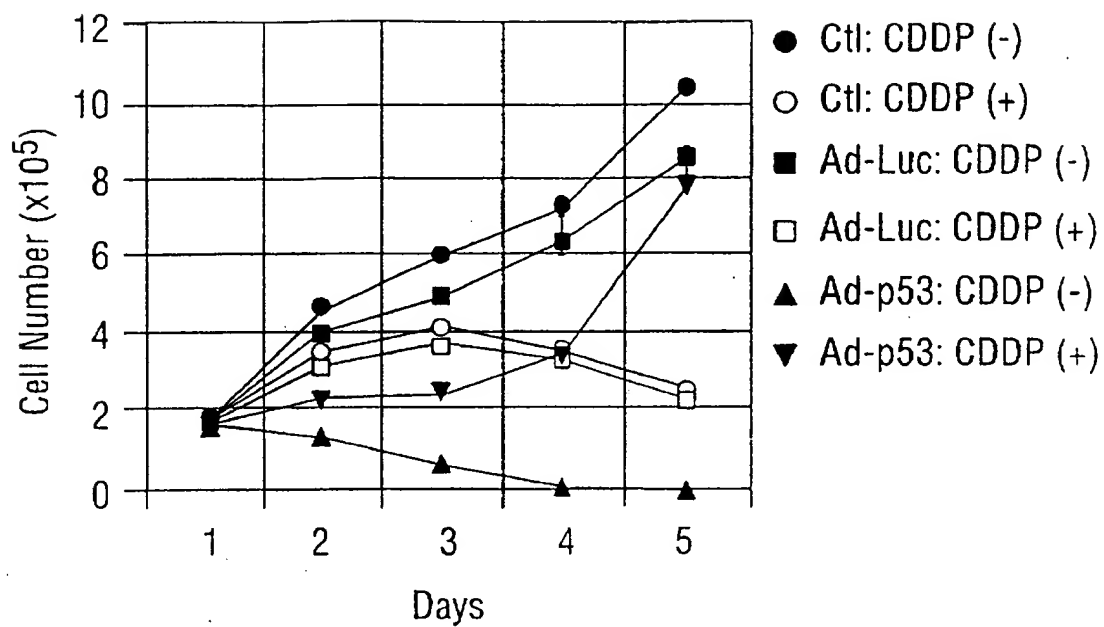


FIG. 10A

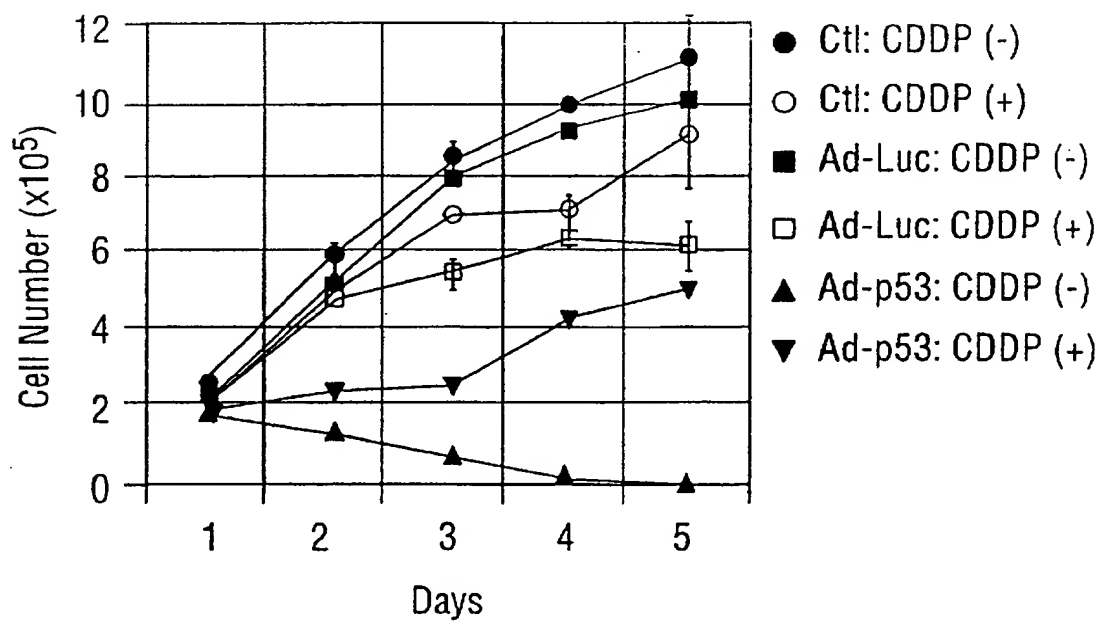


FIG. 10B

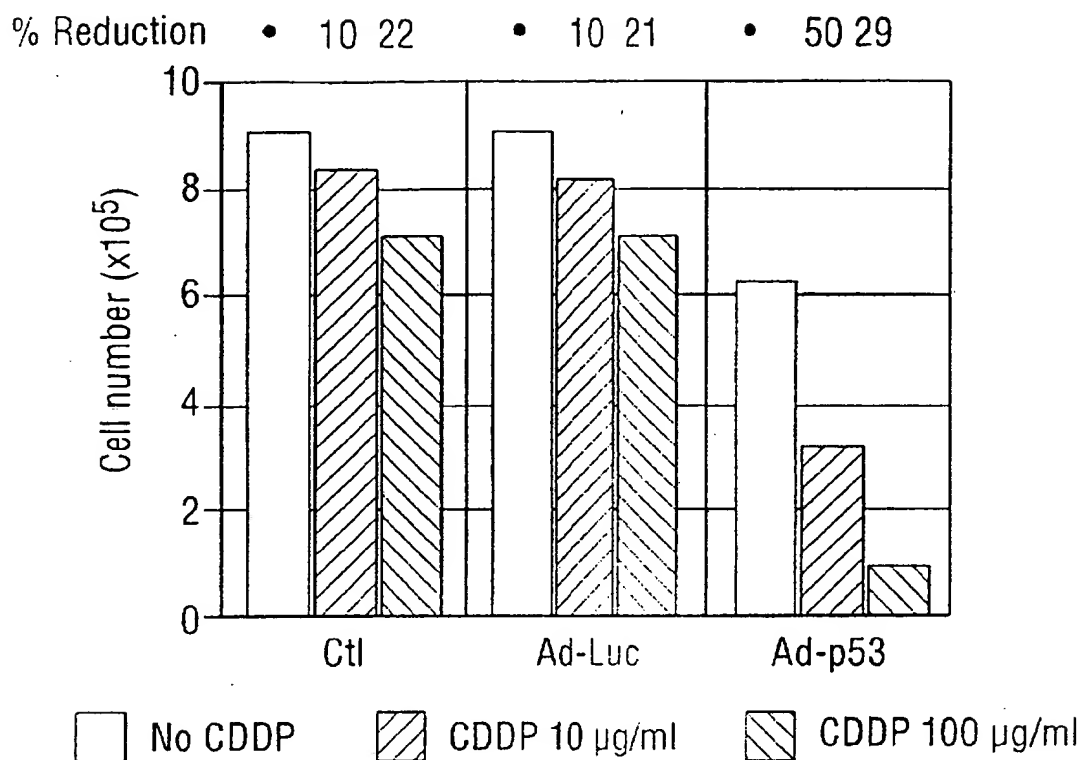


FIG. 10C

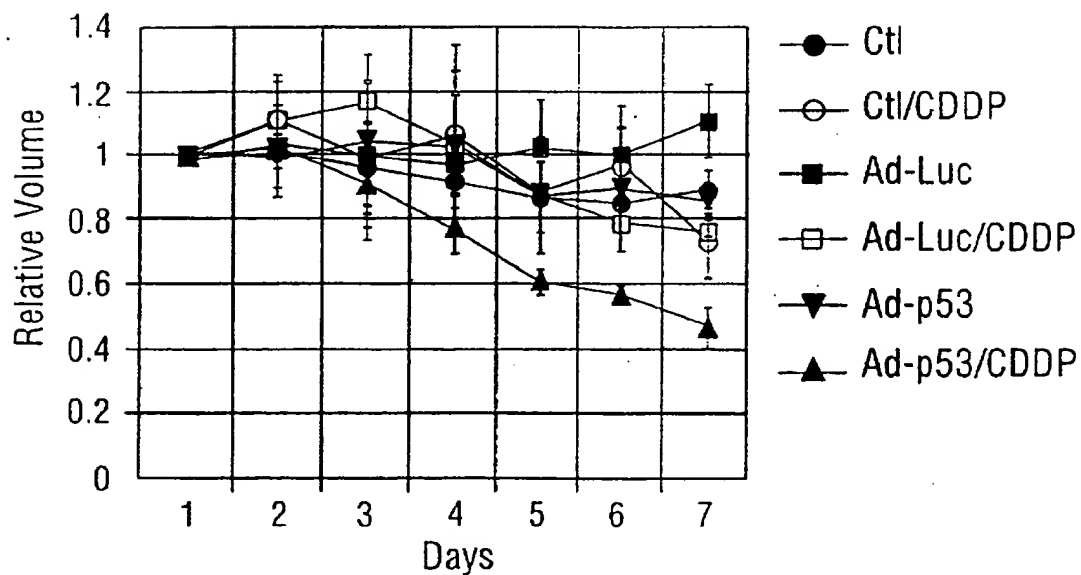


FIG. 12A



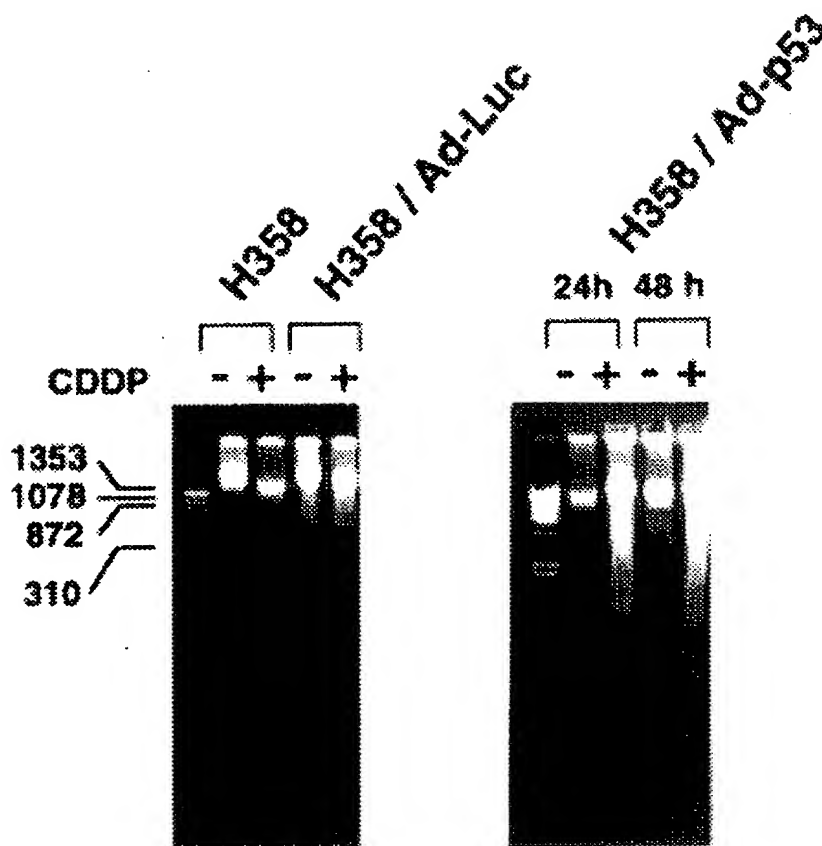


FIG.11A

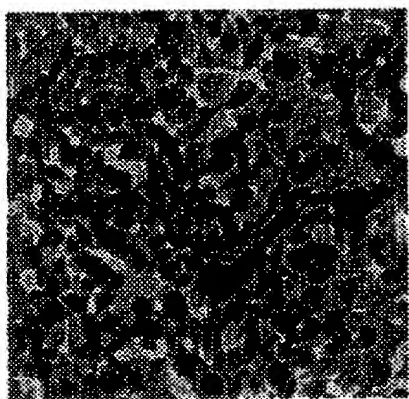


FIG. 11B

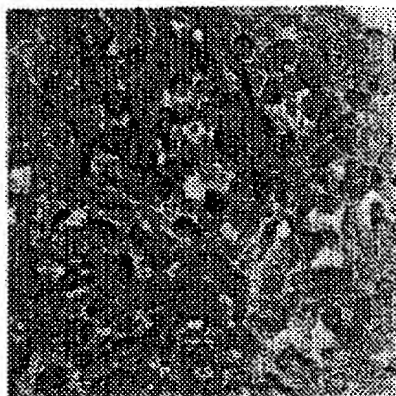


FIG. 11C

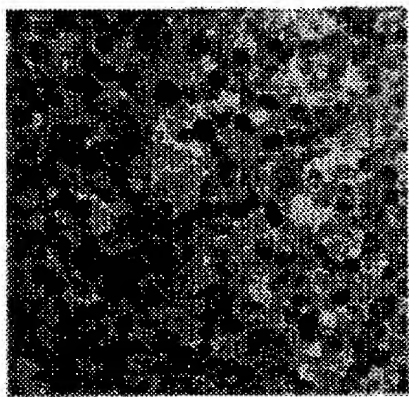


FIG. 11D

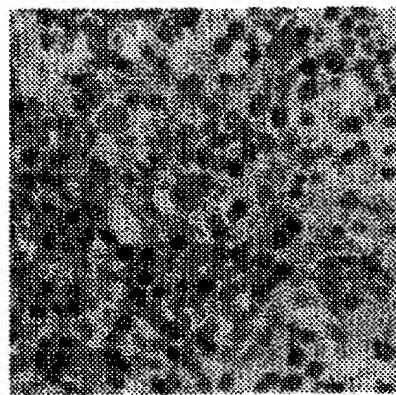


FIG. 11E

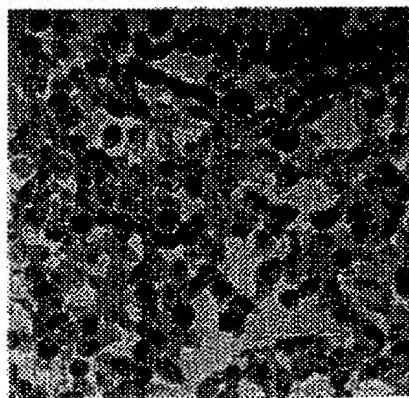


FIG. 11F

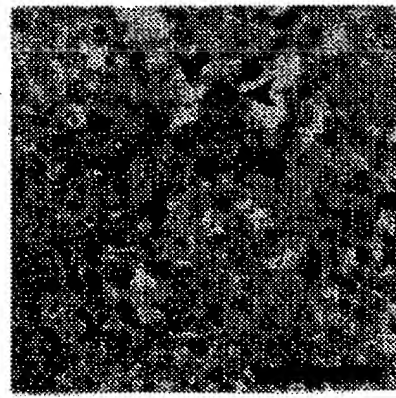


FIG. 11G

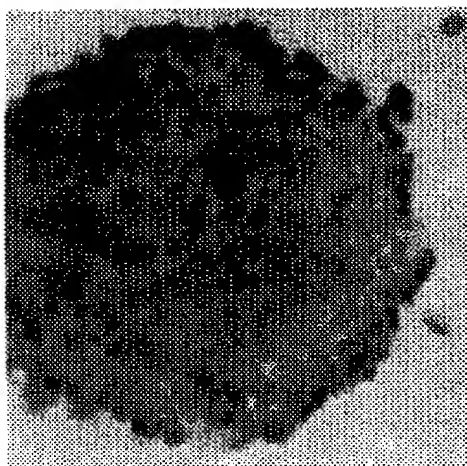


FIG. 12B

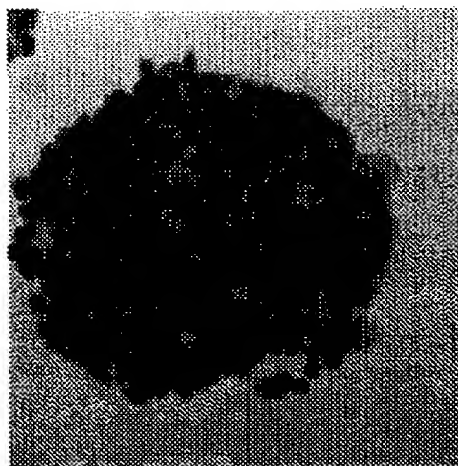


FIG. 12C

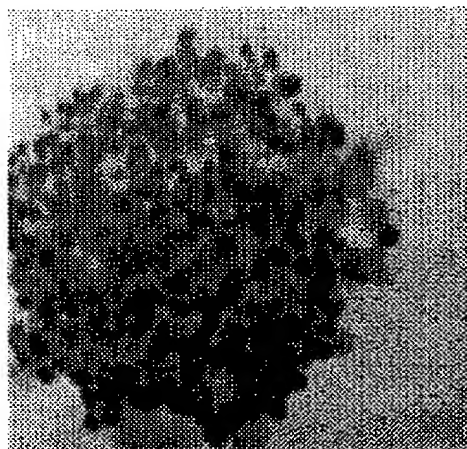


FIG. 12D

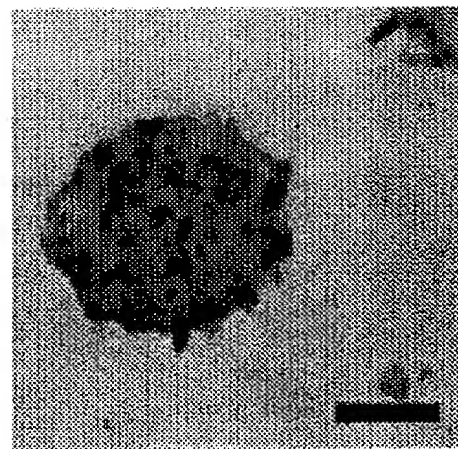


FIG. 12E

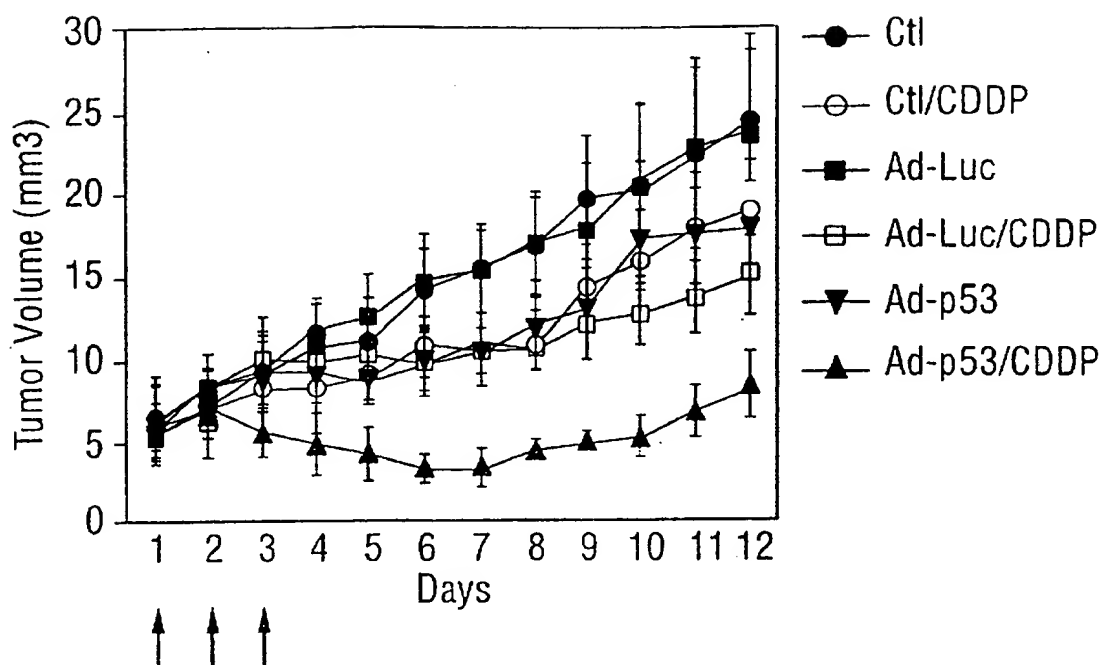


FIG. 13A-1

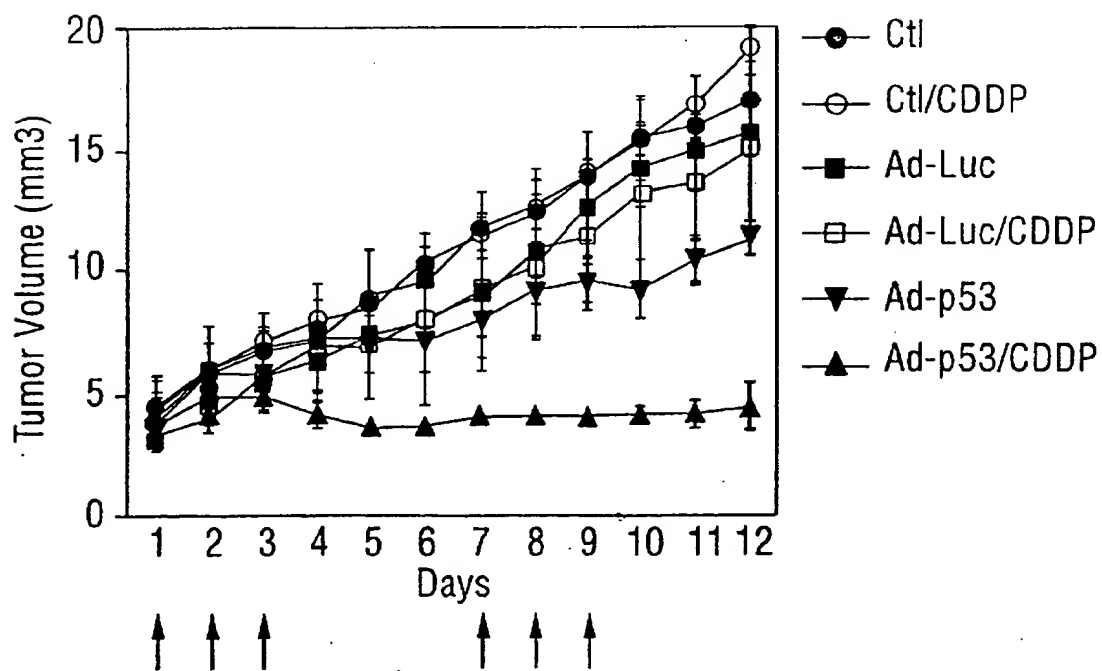


FIG. 13A-2

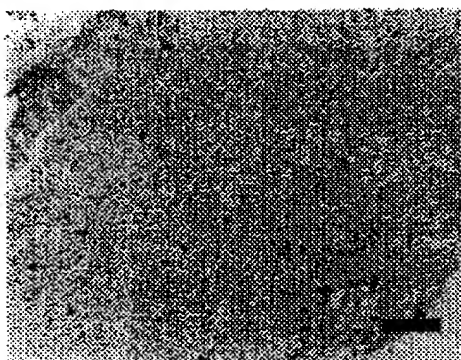


FIG. 13B

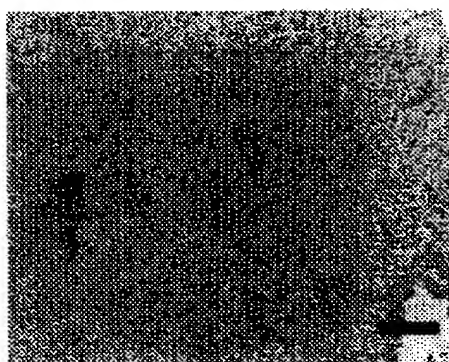


FIG. 13C

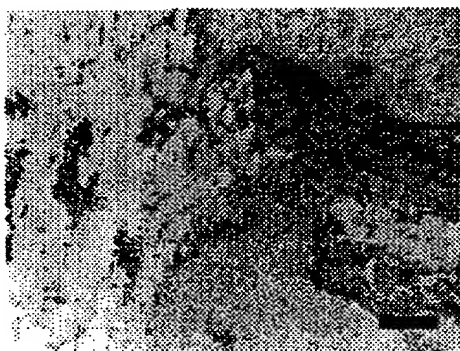


FIG. 13D



FIG. 13E

## METHODS AND COMPOSITIONS COMPRISING DNA DAMAGING AGENTS AND P53

The present application is a continuation-in-part of co-pending U.S. patent application Ser. No. 08/145,826, filed Oct. 29, 1993; which is a continuation-in-part of U.S. patent application Ser. No. 07/960,513, filed Oct. 13, 1992; which is a continuation-in-part of U.S. Ser. No. 07/665,538, filed Mar. 6, 1991 now abandoned; the entire text and figures of which disclosures are incorporated herein by reference without disclaimer.

The government owns rights in the present invention pursuant to NIH grants RO1 CA 45187 and CA 16672, and Training Grants CA 09611 and CA 45225.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates generally to the area of novel strategies for the improvement of chemotherapeutic intervention. In other aspects, the present invention provides novel methods and compositions that combine the potency of DNA damaging agents with the combined delivery of a tumor suppressor. The combination of DNA damaging factors with the heterologous expression of a tumor suppressor gene lead to a pronounced synergy over and above the actions of the individual components.

#### 2. Description of Related Art

Current treatment methods for cancer, including radiation therapy, surgery, and chemotherapy, are known to have limited effectiveness. Lung cancer alone kills more than 140,000 people annually in the United States. Recently, age-adjusted mortality from lung cancer has surpassed that from breast cancer in women. Although implementation of smoking-reduction programs has decreased the prevalence of smoking, lung cancer mortality rates will remain high well into the 21st century. The rational development of new therapies for lung cancer will depend on an understanding of the biology of lung cancer at the molecular level.

It is now well established that a variety of cancers are caused, at least in part, by genetic abnormalities that result in either the over expression of one or more genes, or the expression of an abnormal or mutant gene or genes. For example, in many cases, the expression of oncogenes is known to result in the development of cancer. "Oncogenes" are genetically altered genes whose mutated expression product somehow disrupts normal cellular function or control (Spandidos et al., 1989).

Most oncogenes studied to date have been found to be "activated" as the result of a mutation, often a point mutation, in the coding region of a normal cellular gene, i.e., a "proto-oncogene", that results in amino acid substitutions in the expressed protein product. This altered expression product exhibits an abnormal biological function that takes part in the neoplastic process (Travali et al., 1990). The underlying mutations can arise by various means, such as by chemical mutagenesis or ionizing radiation. A number of oncogenes and oncogene families, including ras, myc, neu, raf, erb, src, fms, jun and abl, have now been identified and characterized to varying degrees (Travali et al., 1990; Bishop, 1987).

During normal cell growth, it is thought that growth-promoting proto-oncogenes are counterbalanced by growth-constraining tumor suppressor genes. Several factors may contribute to an imbalance in these two forces, leading to the neoplastic state. One such factor is mutations in tumor suppressor genes (Weinberg, 1991).

An important tumor suppressor gene is the gene encoding the cellular protein, p53, which is a 53 kD nuclear phosphoprotein that controls cell proliferation. Mutations to the p53 gene and allele loss on chromosome 17p, where this gene is located, are among the most frequent alterations identified in human malignancies. The p53 protein is highly conserved through evolution and is expressed in most normal tissues. Wild-type p53 has been shown to be involved in control of the cell cycle (Mercer, 1992), transcriptional regulation (Fields et al., 1990, and Mietz et al., 1992), DNA replication (Wilcock and Lane, 1991, and Bargonetti et al., 1991), and induction of apoptosis (Yonish-Rouach et al., 1991, and, Shaw et al., 1992).

Various mutant p53 alleles are known in which a single base substitution results in the synthesis of proteins that have quite different growth regulatory properties and, ultimately, lead to malignancies (Hollstein et al., 1991). In fact, the p53 gene has been found to be the most frequently mutated gene in common human cancers (Hollstein et al., 1991; Weinberg, 1991), and is particularly associated with those cancers linked to cigarette smoke (Hollstein et al., 1991; Zakut-Houri et al., 1985). The overexpression of p53 in breast tumors has also been documented (Casey et al., 1991).

One of the most challenging aspects of gene therapy for cancer relates to utilization of tumor suppressor genes, such as p53. It has been reported that transfection of wild-type p53 into certain types of breast and lung cancer cells can restore growth suppression control in cell lines (Casey et al., 1991; Takahasi et al., 1992). Although DNA transfection is not a viable means for introducing DNA into patients' cells, these results serve to demonstrate that supplying wild type p53 to cancer cells having a mutated p53 gene may be an effective treatment method if an improved means for delivering the p53 gene could be developed.

Gene delivery systems applicable to gene therapy for tumor suppression are currently being investigated and developed. Virus-based gene transfer vehicles are of particular interest because of the efficiency of viruses in infecting actual living cells, a process in which the viral genetic material itself is transferred. Some progress has been made in this regard as, for example, in the generation of retroviral vectors engineered to deliver a variety of genes. However, major problems are associated with using retroviral vectors for gene therapy since their infectivity depends on the availability of retroviral receptors on the target cells, they are difficult to concentrate and purify, and they only integrate efficiently into replicating cells.

Tumor cell resistance to chemotherapeutic drugs represents a major problem in clinical oncology. NSCLC accounts for at least 80% of the cases of lung cancer; patients with NSCLC are, however, generally unresponsive to chemotherapy (Doyle, 1993). One goal of current cancer research is to find ways to improve the efficacy of gene replacement therapy for cancer by investigating interaction between the gene product and chemotherapeutic drugs. The herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, et al., 1992). The HS-tK gene product is an exogenous viral enzyme, whereas the wt-p53 protein is expressed in normal tissues, suggesting that the modulation of chemoresistance by alterations in wt-p53 expression might be an alternative approach using a pathway mediated by an endogenous genetic program.

An adenovirus system has potential advantages for gene delivery in vivo, such as ease of producing high titer virus.

high infection efficiency, and infectivity for many types of cells. The stability and duration of expression of the introduced gene are still controversial, however. The increase in p53 levels in cells that are sensitive to chemotherapeutic drugs can occur within 6 hours after DNA-damaging stimuli (Fritsche, et al., 1993; Zhan, et al., 1993), although increased p53 DNA binding activity can be reversed over the course of 4 hours if the stimulus is removed (Tishler, et al., 1993). Therefore, a high level of p53 expression can be maintained even after cessation of drug exposure. The expression of wt-p53 protein by Ad-p53 peaks at postinfection day 3 (14-fold greater than endogenous wild type) and decreases to a low level by day 9 (Zhang, et al., 1993). This suggests that a transiently high level of wt-p53 expression is sufficient to initiate the cytotoxic program in the cancer cell.

p53 has an important role as a determinant of chemosensitivity in human lung cancer cells. A variety of treatment protocols, including surgery, chemotherapy, and radiotherapy, have been tried for human NSCLC, but the long-term survival rate remains unsatisfactory. What is needed is a combination therapy that is used alone or as an effective adjuvant treatment to prevent local recurrence following primary tumor resection or as a treatment that could be given by intralesional injections in drug-resistant primary, metastatic, or locally recurrent lung cancer. Compositions and methods are also needed to develop, explore and improve clinical applicability of novel compositions for the treatment of cancer. Furthermore these methods and compositions must prove their value in an in vivo setting.

#### SUMMARY OF THE INVENTION

The present invention addresses the need for improved therapeutic preparations for use in killing cells by combining the effects of a tumor suppressor gene or protein and a DNA damaging agent or factor. The present invention also provides compositions and methods, including those that use viral mediated gene transfer, to promote expression of a wild-type tumor suppressor gene, such as p53, in target cells and to deliver an agent or factor that induces DNA damage. The inventors surprisingly found that using the compositions disclosed herein, they were able to induce programmed cell death, also known as apoptosis, in a very significant number of target cells.

Using the present invention the inventors have demonstrated a remarkable effect in controlling cell growth and in particular tumor cell growth. Tumor cell formation and

prostate hyperplasia cells or over-active thyroid cells; cells relating to autoimmune diseases, such as B cells that produce antibodies involved in arthritis, lupus, myasthenia gravis, squamous metaplasia, dysplasia and the like. Although generally applicable to killing all undesirable cells, the invention has a particular utility in killing malignant cells. "Malignant cells" are defined as cells that have lost the ability to control the cell division cycle, as leads to a "transformed" or "cancerous" phenotype.

To kill cells, such as malignant or metastatic cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with a p53 protein or gene and at least one DNA damaging agent in a combined amount effective to kill the cell. This process may involve contacting the cells with the p53 protein or gene and the DNA damaging agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the p53 protein or gene and the other includes the DNA damaging agent.

Naturally, it is also envisioned that the target cell may be first exposed to the DNA damaging agent(s) and then contacted with a p53 protein or gene, or vice versa. However, in embodiments where the DNA damaging factor and p53 are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the DNA damaging agent and p53 would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both agents within about 12-24 hours of each other, and more preferably within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred.

The terms "contacted" and "exposed", when applied to a cell, are used herein to describe the process by which a tumor suppressor gene or protein, such as p53, and a DNA damaging agent or factor are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell, i.e., to induce programmed cell death or apoptosis. The terms, "killing", "programmed cell death" and "apoptosis" are used interchangeably in the present text to describe a series of intracellular events that lead to target cell death. The process of cell death involves the activation of intracellular proteases and nucleases that



compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. In certain embodiments, the use of cisplatin in combination with a p53 protein or gene is particularly preferred as this compound.

Any method may also be used to contact a cell with a p53 protein, so long as the method results in increased levels of functional p53 protein within the cell. This includes both the direct delivery of a p53 protein to the cell and the delivery of a gene or DNA segment that encodes p53, which gene will direct the expression and production of p53 within the cell. In that protein delivery is subject to such drawbacks as protein degradation and low cellular uptake, it is contemplated that the use of a recombinant vector that expresses a p53 protein will provide particular advantages.

A wide variety of recombinant plasmids and vectors may be engineered to express a p53 protein and so used to deliver p53 to a cell. These include, for example, the use of naked DNA and p53 plasmids to directly transfer genetic material into a cell (Wolfe et al., 1990); formulations of p53-encoding DNA trapped in liposomes (Ledley et al., 1987) or in proteoliposomes that contain viral envelope receptor proteins (Nicolau et al., 1983); and p53-encoding DNA coupled to a polylysine-glycoprotein carrier complex.

The use of recombinant viruses engineered to express p53 is also envisioned. A variety of viral vectors, such as retroviral vectors, herpes simplex virus (U.S. Pat. No. 5,288,641, incorporated herein by reference), cytomegalovirus, and the like may be employed, as described by Miller (Miller, 1992); as may recombinant adeno-associated virus (AAV vectors), such as those described by U.S. Pat. No. 5,139,941, incorporated herein by reference; and, particularly, recombinant adenoviral vectors. Techniques for preparing replication-defective infective viruses are well known in the art, as exemplified by Ghosh-Choudhury & Graham (1987); McGrory et al. (1988); and Gluzman et al. (1982), each incorporated herein by reference.

To kill a cell in accordance with the present invention, one would generally contact the cell with a p53 protein or gene and a DNA damaging agent in a combined amount effective to kill the cell. The term "in a combined amount effective to kill the cell" means that the amount of p53 and DNA damaging agents are sufficient so that, when combined within the cell, the cell is induced to undergo apoptosis. Although not required in all embodiments, the combined effective amount of p53 and DNA damaging agent will preferably be an amount that induces significantly more cell death than the use of either element alone, and most preferably, the combined effective amount will be an amount that induces synergistic cell death in comparison to the effects observed using either element alone.

A number of in vitro parameters may be used to determine the effect produced by the compositions and methods of the present invention. These parameters include, for example, the observation of net cell numbers before and after exposure to the compositions described herein, as well as the size of multicellular tumor spheroids formed, such as those colonies formed in tissue culture. In vitro cell killing is particularly shown in Example 7 of the present disclosure. Alternatively, one may measure parameters that are indicative of a cell that is undergoing programmed cell death, such as, the fragmentation of cellular genomic DNA into nucleosome size fragments, generally identified by separating the fragments by agarose gel electrophoresis, staining the DNA, and comparing the DNA to a DNA size ladder. Nucleosome size fragments are identified as a progressive steps or ladders of monomers and multimers having a base unit of about 200 basepairs.

Similarly, a "therapeutically effective amount" is an amount of a p53 protein or gene and DNA damaging agent that, when administered to an animal in combination, is effective to kill cells within the animal. This is particularly evidenced by the killing of cancer cells, such as lung, breast or colon cancer cells, within an animal or human subject that has a tumor. "Therapeutically effective combinations" are thus generally combined amounts of p53 and DNA damaging agents that function to kill more cells than either element alone, and preferably, combined amounts that bring about a synergistic reduction in tumor burden.

Studying certain in vivo and ex vivo parameters of cell death are therefore also effective means by which to assess the effectiveness of the composition and methods of the invention. For example, observing effects on the inhibition of tumorigenicity, as measured by TdT expression of frozen tissue sections or by using other staining methods and target antigens, as known to skilled pathologists. Naturally, other means of determining tumor mass, growth, and viability may also be used to assess the killing of target cells. In particular, one may assess the effects in various animal model systems of cancer, including those in which human cancer cells are localized within the animal. Animal models of cancer, unlike those of AIDS, are known to be highly predictive of human treatment regimens (Roth et al., editors (1989)). One exemplary embodiment of a predictive animal model is that in which human small-cell lung cancer cells (H358 cells) are grown subcutaneously. Using this system, the inventors have shown that p53-bearing adenovirus instilled intratumorally, along with the co-administration of a chemotherapeutic agent, gives rise to a surprisingly effective tumor reduction.

A particularly preferred method of delivering a p53 protein to a cell is to contact the cell with a recombinant adenovirus virion or particle that includes a recombinant adenoviral vector comprising a p53 expression region positioned under the control of a promoter capable of directing the expression of p53 in the given cell type.

The p53 expression region in the vector may comprise a genomic sequence, but for simplicity, it is contemplated that one will generally prefer to employ a p53 cDNA sequence as these are readily available in the art and more easily manipulated. In addition to comprising a p53 expression unit and a promoter region, the vector will also generally comprise a polyadenylation signal, such as an SV40 early gene, or protamine gene, polyadenylation signal, or the like.

In preferred embodiments, it is contemplated that one will desire to position the p53 expression region under the control of a strong constitutive promoter such as a CMV promoter, viral LTR, RSV, or SV40 promoter, or a promoter associated with genes that are expressed at high levels in mammalian cells such as elongation factor-1 or actin promoters. All such variants are envisioned to be useful with the present invention. Currently, a particularly preferred promoter is the cytomegalovirus (CMV) IE promoter.

The p53 gene or cDNA may be introduced into a recombinant adenovirus in accordance with the invention simply by inserting or adding the p53 coding sequence into a viral genome. However, the preferred adenoviruses will be replication defective viruses in which a viral gene essential for replication and/or packaging has been deleted from the adenoviral vector construct, allowing the p53 expression region to be introduced in its place. Any gene, whether essential (e.g., E1A, E1B, E2 and E4) or non-essential (e.g., E3) for replication, may be deleted and replaced with p53. Particularly preferred are those vectors and virions in which



the E1A and E1B regions of the adenovirus vector have been deleted and the p53 expression region introduced in the place, as exemplified by the genome structure of FIG. 1.

Techniques for preparing replication defective adenoviruses are well known in the art, as exemplified by Ghosh-Choudhury and Graham (1987); McGrory et al. (1988); and Gluzman et al., each incorporated herein by reference. It is also well known that various cell lines may be used to propagate recombinant adenoviruses, so long as they complement any replication defect which may be present. A preferred cell line is the human 293 cell line, but any other cell line that is permissive for replication, i.e., in the preferred case, which expresses E1A and E1B may be employed. Further, the cells can be propagated either on plastic dishes or in suspension culture, in order to obtain virus stocks thereof.

The invention is not limited to E1-lacking virus and E1-expressing cells alone. Indeed, other complementary combinations of viruses and host cells may be employed in connection with the present invention. Virus lacking functional E2 and E2-expressing cells may be used, as may virus lacking functional E4 and E4-expressing cells, and the like. Where a gene which is not essential for replication is deleted and replaced, such as, for example, the E3 gene, this defect will not need to be specifically complemented by the host cell.

Other than the requirement that the adenovirus vectors be engineered to express p53, the nature of the initial adenovirus is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which there is significant amount of biochemical and genetic information known, and which has historically been used for most constructions employing adenovirus as a vector.

The methods and compositions of the present invention are equally suitable for killing a cell or cells both in vitro and in vivo. When the cells to be killed are located within an animal, e.g., lung, breast or colon cancer cells or other cells bearing a p53 mutation, both the p53 protein or gene and the DNA damaging agent will be administered to the animal in a pharmacologically acceptable form. The term "a pharmacologically acceptable form", as used herein, refers to both the form of any composition that may be administered to an animal, and also the form of contacting an animal with radiation, i.e., the manner in which an area of the animal's body is irradiated, e.g., with  $\gamma$ -irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. The use of DNA damaging radiation and waves is known to those skilled in the art of irradiation therapy.

The present invention also provides advantageous methods for treating cancer that, generally, comprise administering to an animal or human patient with cancer a therapeutically effective combination of a p53 protein or gene and a DNA damaging agent. This may be achieved using a recombinant virus, particularly an adenovirus, that carries a vector capable of expressing p53 in the cells of the tumor. The p53 gene delivering composition would generally be administered to the animal, often in close contact to the tumor, in the form of a pharmaceutically acceptable composition. Direct intraslesional injection of a therapeutically effective amount of a p53 gene, such as housed within a recombinant virus,

into a tumor site is one preferred method. However, other parenteral routes of administration, such as intravenous, percutaneous, endoscopic, or subcutaneous injection are also contemplated.

In treating cancer according to the invention one would contact the tumor cells with a DNA damaging agent in addition to the p53 protein or gene. This may be achieved by irradiating the localized tumor site with DNA damaging radiation such as X-rays, UV-light,  $\gamma$ -rays or even microwaves. Alternatively, the tumor cells may be contacted with the DNA damaging agent by administering to the animal a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging compound, such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. The DNA damaging agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a p53 protein, gene or gene delivery system, as described above.

The surprising success of the present invention is evidenced by the finding that using Ad5CMV-p53 virus in combination with cisplatin yielded profound results in studies using a nude mouse model. The combined virus-DNA damage therapy regimen significantly inhibited the tumorigenicity of H358 cells, a cell that normally produces a significant tumor mass. The tumorigenicity of the lung cancer cells was inhibited through the treatment by Ad5CMV-p53, but not by the control virus expressing luciferase, indicating that the p53 protein in combination with a DNA-damaging agent has great therapeutic efficacy.

A number of methods for delivering chemotherapeutic formulations, including DNA expression constructs, into eukaryotic cells are known to those of skill in the art. In light of the present disclosure, the skilled artisan will be able to deliver both DNA damaging agents and p53 proteins or genes to cells in many different effective ways.

For in vivo delivery of DNA, the inventors envision the use of any gene delivery system, such as viral- and liposome-mediated transfection. As used herein, the term "transfection", is used to describe the targeted delivery of DNA to eukaryotic cells using delivery systems, such as, adenoviral, AAV, retroviral, or plasmid delivery gene transfer methods. The specificity of viral gene delivery may be selected to preferentially direct the gene to a particular target cell, such as by using viruses that are able to infect particular cell types. Naturally, different viral host ranges will dictate the virus chosen for gene transfer, as well as the likely tumor suppressor gene to be expressed for killing a given malignant cell type.

It is also envisioned that one may provide the DNA damaging chemotherapeutic agent through a variety of means, such as by using parenteral delivery methods such as intravenous and subcutaneous injection, and the like. Such methods are known to those of skill in the art of drug delivery, and are further described herein in the sections regarding pharmaceutical preparations and treatment.

For in vitro gene delivery, a variety of methods may be employed, such as, e.g., calcium phosphate- or dextran sulfate-mediated transfection; electroporation; glass projectile targeting; and the like. These methods are known to those of skill in the art, with the exact compositions and execution being apparent in light of the present disclosure.

Other embodiments concern compositions, including pharmaceutical formulations, comprising a p53 protein or gene in combination with a DNA damaging agent, such as cisplatin. In such compositions, the p53 may be in the form

a DNA segment, recombinant vector or recombinant virus that is capable of expressing a p53 protein in an animal cell. These compositions, including those comprising a recombinant viral gene delivery system, such as an adenovirus particle, may be formulated for in vivo administration by dispersion in a pharmacologically acceptable solution or buffer. Preferred pharmacologically acceptable solutions include neutral saline solutions buffered with phosphate, lactate, Tris, and the like.

Of course, in using viral delivery systems, one will desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, animal or individual receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

Preferred pharmaceutical compositions of the invention are those that include, within a pharmacologically acceptable solution or buffer, a p53 protein, or more preferably a p53 gene, in combination with a chemotherapeutic DNA damaging agent. Exemplary chemotherapeutic agents are adriamycin, 5-fluorouracil, camptothecin, actinomycin-D, hydrogen peroxide, mitomycin C, cisplatin (CDDP), and etoposide (VP-16), with the use of cisplatin being particularly preferred.

Still further embodiments of the present invention are kits for use in killing cells, such as malignant cells, as may be formulated into therapeutic kits for use in cancer treatment. The kits of the invention will generally comprise, in suitable container means, a pharmaceutical formulation of a recombinant vector that is capable of expressing a p53 protein in an animal cell, and a pharmaceutical formulation of a DNA damaging agent. The recombinant vectors and DNA damaging agents may be present within a single container, or these components may be provided in distinct or separate container means. In a preferred embodiment, the recombinant vector will be a recombinant p53-expressing adenoviral vector present within an adenovirus particle and the DNA damaging agent will be cisplatin.

The components of the kit are preferably provided as a liquid solution, or as a dried powder. When the components are provided in a liquid solution, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Scheme for generation of recombinant p53 adenovirus. The p53 expression cassette was inserted between the Xba I and Cla I sites of pXCJL.1. The p53 expression vector (pEC53) and the recombinant plasmid pJM17 were cotransfected into 293 cells. The transfected cells were maintained in medium until the onset of the cytopathic effect. Identification of newly generated p53 recombinant adenoviruses (Ad5CMV-p53) by PCR analysis of the DNA using DNA templates prepared from the CPE supernatants treated with Proteinase K and phenol extraction.

FIG. 2A. Map used for the structural analysis of Ad5CMV-p53 DNA. A map of Ad5CMV-p53 genomic DNA, with the locations of the p53 expression cassette, the PCR primers, and the restriction sites. The genome size is about 35.4 kb, divided into 100 maps units (1 m.u.=0.35 kb). The p53 expression cassette replaced the E1 region (1.3-9.2 m.u.) of the Ad5 genome. Primer 1 is located in the first intron downstream of the human CMV major IE gene promoter. Primer 2 is located in SV40 early polyadenylation signal. Both of the primers, 15-20 bp away from the p53 cDNA insert at both ends, define a 1.40 kb PCR product. Primers 3 and 4 are located at 11 m.u. and 13.4 m.u. of Ad5 genome, respectively, which define a 0.86 kb viral-genome specific PCR product.

FIG. 2B. Agarose gel analysis of PCR products. Two pairs of primers that define 1.4-kb (p53) and 0.86-kb (Ad5) DNA fragments were used in each reaction. DNA templates used in each reaction were pEC53 plasmid (lane 1), Ad5/RSV/GL2 DNA (lane 2), no DNA (lane 3), and Ad5CMV-p53 DNA (lane 4). The lane labeled (M) corresponds to molecular weight markers.

FIG. 2C. Restriction mapping of Ad5CMV-p53 DNA. CsCl-gradient purified Ad5CMV-p53 DNA samples were digested with no enzyme (U), Hind III (H), Bam HI (B), Eco RI (E), and Cla I (C), respectively, and analyzed on 1% agarose gel. The lanes labeled (M) are molecular weight markers.

FIGS. 3A, 3B, 3C and 3D. Observation of cytopathic effects on 293 by recombinant adenovirus. FIGS. 3A, 3B, 3C and 3D are a series of phase contrast images ( $\times 400$ ) of 293 cells. FIGS. 3A, 3B, 3C and 3D are four panels of a single page figure. FIG. 2A, before transfection; FIG. 3B, negative control on day 12 posttransfection; FIG. 3C, onset of CPE on day 12 posttransfection; FIG. 3D, completion of CPE on day 14 post-transfection.

FIGS. 4A, 4B, 4C, and 4D. Immunohistology of cells infected with recombinant adenoviruses. FIGS. 4A, 4B, 4C and 4D are a series of immunohistological images of H358 cells. FIGS. 4A, 4B, 4C and 4D are four panels of a single page figure. Infectivity of Ad5CMV-p53 in H358 cells. H358 cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 50 PFU/cell for 24 h. Medium alone was used as a mock infection. The infected cells were analyzed by immunostainings. FIG. 4A is a mock infection probed with anti-p53 antibody. FIG. 4B are cells infected with the Ad5/RSV/GL2 control and probed with anti-p53 antibody. FIG. 4C are Ad5CMV-p53 infected cells probed with an unrelated antibody (MOPC-21). FIG. 4D are cells Ad5CMV-p53 infection probed with anti-p53 antibody. The anti-p53 antibody used was Pab 1801, and the avidin-biotin method was used for staining.

FIG. 5A. Coomassie-blue stained SDS-PAGE gel comparing the relative level of expression of exogenous p53 in H358 cells. H358 cell samples that were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 30 PFU/cell were prepared 24 and 72 h after infection. Coomassie blue staining of an SDS-PAGE analysis, showing relative quantities of protein samples loaded. Lanes 1 and 4 contain the samples of the Ad5/RSV/GL2-infected cells. Lanes 2 and 3 contain the samples of the cells infected with two individual stocks of Ad5CMV-p53 at 24 h after infection. Lanes 5 and 6 are the Ad5CMV-p53-infected cell samples collected at 72 h after infection. Lane 7 is mock-infected H358 sample 72 h after infection. Lane M, prestained molecular weight markers in kDa (GIBCO-BRL).

FIG. 5B. Western blot analysis of the identical lane setting gel as that of the SDS-PAGE in FIG. 5A. The relative levels

of expression of p53 were analyzed by Western blotting using anti-p53. Primary antibodies used were monoclonal antibodies against p53 protein (PAB 1801, Oncogene Science Inc.) and  $\beta$ -actin (Amersham Inc.). The HRP-conjugated second antibody and ECL developer were from Amersham Inc. viral-infected H358 cells by Western Blotting. Western blot of FIG. 5B have an equivalent setup and order to those in FIG. 5A.

FIGS. 6A-B. Time course of the p53 expression, determined by Western blotting (FIG. 6B). Multiple dishes of H358 cells were infected with Ad5CMV-p53 at 10 PFU/cell. Cell lysates were prepared at indicated time points after infection. Western blotting was probed with anti-p53 and anti-actin antibodies simultaneously. The lanes designated 'C' represent negative controls. The histogram represents the relative quantities of p53 as determined by a densitometer (FIG. 6A).

FIG. 7A. Growth curve of virally-infected human lung cancer cells of cell lines H358. Cells were inoculated at  $10^5$  cells per dish (60 mm) and 6 dishes per cell line. After 24 hours, the cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 m.o.i. (Multiplicity of infection, i.e., PFU/cell). After infection cells were counted daily for 6 days. The growth curves represent data obtained from 4 separate studies.

FIG. 7B. Growth curve of virally-infected human lung cancer cells of cell line H322. Cells were inoculated at  $10^5$  cells per dish (60 mm) and 6 dishes per cell line. After 24 hours, the cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 m.o.i. (Multiplicity of infection, i.e., PFU/cell). After infection cells were counted daily for 6 days. The growth curves represent data obtained from 4 separate studies.

FIG. 7C. Growth curve of virally-infected human lung cancer cells of cell line H460. Cells were inoculated at  $10^5$  cells per dish (60 mm) and 6 dishes per cell line. After 24 hours, the cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 m.o.i. (Multiplicity of infection, i.e., PFU/cell). After infection cells were counted daily for 6 days. The growth curves represent data obtained from 4 separate studies.

FIG. 8. Flow chart of tests of Ad5CMV-p53 in orthotopic lung cancer model. The dosages and schedule of treatment of nude mice inoculated with H226Br cells and viruses are summarized in the flow chart.

FIGS. 9A, 9B, 9C, and 9D. Samples of the lung and mediastinum dissection from treated and control mice. FIGS. 9A, 9B, 9C, and 9D are four panels of a single figure. The mice were sacrificed at the end of the 6-week posttreatment period. The lung and mediastinum tissues were dissected for evaluation of tumor formation. FIG. 9A is a sample of mediastinal block from a normal nude mice; FIG. 9B is the mediastinal block sample from the vehicle (PBS)-treated mice; FIG. 9C is the mediastinal block sample from the Ad5CMV-p53-treated mice; FIG. 9D is the mediastinal block sample from the Ad5/RSV/GL2-treated mice. Arrows indicate the tumor masses.

FIG. 10A. The effects of continuous exposure to CDDP on the growth rates of parental, Ad-Luc-infected, and Ad-p53-infected H358 cells. H358 cells ( $1.5 \times 10^5$  cells/well) were seeded in duplicate on a 24-well plate. After 24 hours, 100  $\mu$ l of medium, Ad-Luc viral stock ( $10^8$  PFU/ml), or Ad-p53 viral stock ( $10^8$  PFU/ml) was added. Following an additional 24-hour incubation, the medium that contained virus was replaced with fresh medium that contained 10  $\mu$ g/ml of CDDP.

FIG. 10B. 24-hour exposure to CDDP on the growth rates of parental, Ad-Luc-infected, and Ad-p53-infected H358 cells. Cells were exposed to CDDP (FIG. 10A) continuously or (FIG. 10B) for 24 hours followed by recovery in drug-free medium. Cells that remained as an attached monolayer were assessed for viability over 5 days by measuring trypan blue uptake. The mean  $\pm$  SE is shown. The day 5 cell number for the Ad-p53:CDDP group differs significantly from all other groups for both A and B ( $p < 0.05$  by Student's t-test).

FIG. 10C. The effects of different concentrations of CDDP on the viability of Ad-p53-infected H358 cells. After 24-hour exposure to the Ad-Luc or Ad-p53 virus, cells were treated with 0, 10, or 100  $\mu$ g/ml of CDDP for 24 hours and then assessed for viability.

FIG. 11A. Nucleosomal DNA fragmentation in Ad-p53-infected H358 cells exposed to CDDP. Cells were infected and treated with CDDP for 24 hours as described in the legend to FIG. 10.

FIGS. 11B, 11C, 11D, 11E, 11F and 11G. H358 cells that were grown on chamber slides, infected with Ad-p53 for 24 hours, treated with CDDP for an additional 24 hours, and fixed for in situ labeling of DNA fragmentation. Pictured are parental H358 cells (B) without or (C) with CDDP; Ad-Luc-infected cells (D) without or (E) with CDDP; and Ad-p53-infected cells (F) without or (G) with CDDP. The arrowhead shows an example of darkly stained nuclear fragments. Bar=100  $\mu$ m.

FIG. 12A. Effect of the combination of Ad-p53 infection with CDDP treatment on H358 tumor spheroids. Multicellular tumor spheroids of H358 cells were prepared as previously described (Takahashi, et al. (1989)). On day 0, spheroids with a diameter of 150 to 200  $\mu$ m were placed in a 24-well agar coated plate and exposed to Ad-p53 or Ad-Luc for 24 hours. On day 1, medium with 10  $\mu$ g/ml of CDDP was added following removal of virus-containing medium. On day 2, after a 24-hour incubation, the overlay was replaced with 1 ml of fresh drug-free medium. The perpendicular diameters were measured using an inverted microscope. The relative volume change was calculated according to the formula  $a^2 \times b / a_1^2 \times b_1$ , where  $a$  and  $b$  are the smallest and largest diameters of the spheroid, respectively, and  $a_1$  and  $b_1$  are the diameters on day 1. Only the relative volume of the Ad-p53/CDDP spheroids is significantly less ( $p < 0.05$  by Student's t-test) than the control group (Ctl).

FIGS. 12B, 12C, 12D, and 12E. In situ dUTP labeling with TdT for detection of apoptosis. H358 spheroids were fixed on day 3 and stained as described in Materials and Methods of Example 7. (B) Control untreated spheroid, (C) spheroid treated with CDDP, (D) Ad-p53-infected spheroid, and (E) Ad-p53-infected spheroid treated with CDDP. Bar=100  $\mu$ m.

FIGS. 13A-1, 13A-2. Induction of apoptosis by CDDP after in vivo infection with Ad-p53 as measured by tumor volume changes. H358 cells ( $5 \times 10^6$ ) in 0.1 ml Hank's balanced salt solution were injected subcutaneously into the right flank of BALB/c female nu/nu mice. Thirty days later, 200  $\mu$ l of medium alone or medium containing Ad-Luc ( $10^8$  PFU/ml) or Ad-p53 ( $10^8$  PFU/ml) was injected into tumors with a diameter of 5 to 6 mm. Intratumoral injection (100  $\mu$ l) and peritumoral injection in two opposite sites (50  $\mu$ l each) were performed. CDDP (3 mg/kg) or control physiological saline was given intraperitoneally. The tumors were measured with calipers in two perpendicular diameters without the knowledge of the treatment groups, and a tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the

product of cross-sectional diameters. Five mice were used for each treatment group and the mean  $\pm$  SE is shown. The data was analyzed using the Student's t-test. The arrow shows the day of treatment. Two independent determinations are shown.  $p < 0.05$  from day 5 in test 1;  $p < 0.05$  from day 7 in test 2. (B-E)

FIGS. 13B, 13C, 13D, and 13E. Histologic study using the TdT-mediated biotin-dUTP labeling technique. Tumors were harvested 5 days after the beginning of treatment and immediately embedded into O. C. T. compound. Frozen tissues were cut in a cryostat at 5- $\mu$ m thicknesses. The sections were treated with 1  $\mu$ g/ml proteinase K and stained as described in the legend to FIG. 12. Pictured are d to FIG. 12. Pictured are H358 tumors treated with (B) CDDP alone, (C) Ad-p53 alone, or (D, E) Ad-p53 in the combination with CDDP. Bars=0.5 mm. All animal care was in accordance with the UT M. D. Anderson Institutional Animal Care and Use Committee.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### A. Molecular Events in Lung Cancer Development

Studies carried out by the present inventors has identified critical molecular events leading to the development and progression of cancer. This enabled the inventors to develop new methods for restoring certain normal protein functions so that the malignant phenotype can be suppressed in vivo.

The most common lung cancer histologies (80%) are grouped under the term non-small-cell lung cancer (NSCLC) and include squamous, adenocarcinoma, and large-cell undifferentiated. Many of the current data on the molecular biology of lung cancer come from the study of the more uncommon small-cell lung cancer (SCLC). SCLC can be distinguished from NSCLC by the neuroendocrine features of the cells; SCLC is very responsive to chemotherapy but recurs rapidly after treatment. NSCLC also may serve as a model for other carcinogen-induced epithelial cancers. The approaches and observations developed in this study may be applicable to other types of epithelial cancers.

Abundant evidence has accumulated that the process of malignant transformation is mediated by a genetic paradigm. The major lesions detected in cancer cells occur in dominant oncogenes and tumor suppressor genes. Dominant oncogenes have alterations in a class of genes called proto-oncogenes, which participate in critical normal cell functions, including signal transduction and transcription. Primary modifications in the dominant oncogenes that confer the ability to transform include point mutations, translocations, rearrangements, and amplification. Tumor suppressor genes appear to require homozygous loss of function, by mutation, deletion, or a combination of these for transformation to occur. Some tumor suppressor genes appear to play a role in the governance of proliferation by regulation of transcription. Modification of the expression of dominant and tumor suppressor oncogenes is likely to influence certain characteristics of cells that contribute to the malignant phenotype.

Despite increasing knowledge of the mechanisms involved in oncogene-mediated transformation, little progress has occurred in developing therapeutic strategies that specifically target oncogenes and their products. Initially, research in this area was focused on dominant oncogenes, as these were the first to be characterized. DNA-mediated gene transfer studies showed acquisition of the malignant phenotype by normal cells following the transfer of DNA from malignant human tumors.

##### B. p53 and p53 Mutations in Cancer

p53 is currently recognized as a tumor suppressor gene (Montenarh, 1992). High levels have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses, including SV40. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently-mutated gene in common human cancers (Mercer, 1992). It is mutated in over 50% of human NSCLC (Hollestein et al., 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 375-amino-acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue. Interestingly, wild-type p53 appears to be important in regulating cell growth and division. Overexpression of wild-type p53 has been shown in some cases to be anti-proliferative in human tumor cell lines. Thus p53 can act as a negative regulator of cell growth (Weinberg, 1991) and may directly suppress uncontrolled cell growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild type p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene.

Although wild-type p53 is recognized as a centrally important growth regulator in many cell types, its genetic and biochemical traits appear to have a role as well. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Casey and colleagues have reported that transfection of DNA encoding wild-type p53 into two human breast cancer cell lines restores growth suppression control in such cells (Casey et al., 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, p53 into human lung cancer cell lines (Takahasi et al., 1992). The p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 does not affect the growth of cells with endogenous p53. Thus, such constructs might be taken up by normal cells without adverse effects.

It is thus possible that the treatment of p53-associated cancers with wild type p53 may reduce the number of malignant cells. However, studies such as those described above are far from achieving such a goal, not least because DNA transfection cannot be employed to introduce DNA into cancer cells within a patients' body.

##### C. Gene Therapy Techniques

There have been several experimental approaches to gene therapy proposed to date, but each suffer from their particular drawbacks (Mulligan, 1993). As mentioned above, basic transfection methods exist in which DNA containing the gene of interest is introduced into cells non-biologically, for

example, by permeabilizing the cell membrane physically or chemically. Naturally, this approach is limited to cells that can be temporarily removed from the body and can tolerate the cytotoxicity of the treatment, i.e. lymphocytes. Liposomes or protein conjugates formed with certain lipids and amphophilic peptides can be used for transfection, but the efficiency of gene integration is still very low, on the order of one integration event per 1,000 to 100,000 cells, and expression of transfected genes is often limited to days in proliferating cells or weeks in non proliferating cells. DNA transfection is clearly, therefore, not a suitable method for cancer treatment.

A second approach capitalizes on the natural ability of viruses to enter cells, bringing their own genetic material with them. Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines. However, three major problems hamper the practical use of retrovirus vectors. First, retroviral infectivity depends on the availability of the viral receptors on the target surface. Second, retroviruses only integrate efficiently into replicating cells. And finally, retroviruses are difficult to concentrate and purify.

#### D. Adenovirus Constructs for use in Gene Therapy

Human adenoviruses are double-stranded DNA tumor viruses with genome sizes of approximate 36 kb (Tooza, 1981). As a model system for eukaryotic gene expression, adenoviruses have been widely studied and well characterized, which makes them an attractive system for development of adenovirus as a gene transfer system. This group of viruses is easy to grow and manipulate, and they exhibit a broad host range in vitro and in vivo. In lytically infected cells, adenoviruses are capable of shutting off host protein synthesis, directing cellular machineries to synthesize large quantities of viral proteins, and producing copious amounts of virus.

The E1 region of the genome includes E1A and E1B which encode proteins responsible for transcription regulation of the viral genome, as well as a few cellular genes. E2 expression, including E2A and E2B, allows synthesis of viral replicative functions, e.g. DNA-binding protein, DNA polymerase, and a terminal protein that primes replication. E3 gene products prevent cytolysis by cytotoxic T cells and tumor necrosis factor and appear to be important for viral propagation. Functions associated with the E4 proteins include DNA replication, late gene expression, and host cell shutoff. The late gene products include most of the virion capsid proteins, and these are expressed only after most of the processing of a single primary transcript from the major late promoter has occurred. The major late promoter (MLP) exhibits high efficiency during the late phase of the infection (Stratford-Perricaudet and Perricaudet, 1991a).

As only a small portion of the viral genome appears to be required in cis (Tooza, 1981), adenovirus-derived vectors offer excellent potential for the substitution of large DNA fragments when used in connection with cell lines such as 293 cells. Ad5-transformed human embryonic kidney cell line (Graham, et al., 1977) have been developed to provide the essential viral proteins in trans. The inventors thus reasoned that the characteristics of adenoviruses rendered them good candidates for use in targeting cancer cells in vivo (Grunhaus & Horwitz, 1992).

Particular advantages of an adenovirus system for delivering foreign proteins to a cell include (i) the ability to

substitute relatively large pieces of viral DNA by foreign DNA; (ii) the structural stability of recombinant adenoviruses; (iii) the safety of adenoviral administration to humans; and (iv) lack of any known association of adenoviral infection with cancer or malignancies; (v) the ability to obtain high titers of the recombinant virus; and (vi) the high infectivity of Adenovirus.

Further advantages of adenovirus vectors over retroviruses include the higher levels of gene expression. Additionally, adenovirus replication is independent of host gene replication, unlike retroviral sequences. Because adenovirus transforming genes in the E1 region can be readily deleted and still provide efficient expression vectors, oncogenic risk from adenovirus vectors is thought to be negligible (Grunhaus & Horwitz, 1992).

In general, adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1, and yet still retains its competency for infection. Relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. For example, adenoviruses deleted in both E1 and E3 regions are capable of carrying up to 10 Kb of foreign DNA and can be grown to high titers in 293 cells (Stratford-Perricaudet and Perricaudet, 1991a). Surprisingly persistent expression of transgenes following adenoviral infection has also been reported.

Adenovirus-mediated gene transfer has recently been investigated as a means of mediating gene transfer into eukaryotic cells and into whole animals. For example, in treating mice with the rare recessive genetic disorder ornithine transcarbamylase (OTC) deficiency, it was found that adenoviral constructs could be employed to supply the normal OTC enzyme. Unfortunately, the expression of normal levels of OTC was only achieved in 4 out of 17 instances (Stratford-Perricaudet et al., 1991b). Therefore, the defect was only partially corrected in most of the mice and led to no physiological or phenotypic change. These type of results therefore offer little encouragement for the use of adenoviral vectors in cancer therapy.

Attempts to use adenovirus to transfer the gene for cystic fibrosis transmembrane conductance regulator (CFTR) into the pulmonary epithelium of cotton rats have also been partially successful, although it has not been possible to assess the biological activity of the transferred gene in the epithelium of the animals (Rosenfeld et al., 1992). Again, these studies demonstrated gene transfer and expression of the CFTR protein in lung airway cells but showed no physiologic effect. In the 1991 Science article, Rosenfeld et al. showed lung expression of  $\alpha_1$ -antitrypsin protein but again showed no physiologic effect. In fact, they estimated that the levels of expression that they observed were only about 2% of the level required for protection of the lung in humans, i.e., far below that necessary for a physiologic effect.

The gene for human  $\alpha_1$ -antitrypsin has been introduced into the liver of normal rats by intraportal injection, where it was expressed and resulted in the secretion of the introduced human protein into the plasma of these rats (Jaffe et al., 1992). However, and disappointingly, the levels that were obtained were not high enough to be of therapeutic value.

These type of results do not demonstrate that adenovirus is able to direct the expression of sufficient protein in recombinant cells to achieve a physiologically relevant effect, and they do not, therefore, suggest a usefulness of the

adenovirus system for use in connection with cancer therapy. Furthermore, prior to the present invention, it was thought that p53 could not be incorporated into a packaging cell, such as those used to prepare adenovirus, as it would be toxic. As E1B of adenovirus binds to p53, this was thought to be a further reason why adenovirus and p53 technology could not be combined.

#### E. p53-Adenovirus Constructs and Tumor Suppression

The present invention provides cancer gene therapy with a new and more effective tumor suppressor vector. This recombinant virus exploits the advantages of adenoviral vectors, such as high titer, broad target range, efficient transduction, and non-integration in target cells. In one embodiment of the invention, a replication-defective, helper-independent adenovirus is created that expresses wild type p53 (Ad5CMV-p53) under the control of the human cytomegalovirus promoter.

Control functions on expression vectors are often provided from viruses when expression is desired in mammalian cells. For example, commonly used promoters are derived from polyoma, adenovirus 2 and simian virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used provided there is included the approximately 250 bp sequence extending from the HindIII site toward the BglII site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the included gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., polyoma, adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

The design and propagation of the preferred p53 adenovirus is diagramed in FIG. 1. In connection with this, an improved protocol has been developed for propagating and identifying recombinant adenovirus (discussed below). After identification, the p53 recombinant adenovirus was structurally confirmed by the PCR analysis, as indicated in FIG. 2. After isolation and confirmation of its structure, the p53 adenovirus was used to infect human lung cancer cell line H358, which has a homozygous p53 gene deletion. Western blots showed that the exogenous p53 protein was expressed at a high level (FIG. 4 and FIG. 5) and peaked at day 3 after infection (FIG. 6).

It was also shown in a p53 point mutation cell line H322 that the mutant p53 was down regulated by the expression of the exogenous p53. As an experimental control, a virion (Ad5/RSV/GL2) that had a structural similarity to that of Ad5CMV-p53 was used. This virion contained a luciferase CDNA driven by Rous sarcoma virus LTR promoter in the expression cassette of the virion. Neither p53 expression nor change in actin expression was detected in cells infected by the virion Ad5/RSV/GL2. Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in contrast to that of noninfected cells or the cells infected with the control virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the p53 virion (FIG. 7B), while that of human

lung cancer H460 cells containing wild-type p53 was less affected (FIG. 7C).

Ad5CMV-p53 mediated a strong inhibitory effect on lung cancer cell growth in vitro. Growth inhibition was not as evident when the cells were infected with Ad5CMV-p53 at MOI lower than 1 PFU/cell, whereas, at MOI higher than 100 PFU/cell, cytotoxicity could be observed even with control virus Ad5/RSV/GL2. In our studies, the optimal dose for growth rate studies was 10–50 PFU/cell. Within this dose range, cell growth inhibition was attributable to the expressed p53 protein.

Tests in nude mice demonstrated that tumorigenicity of the Ad5CMV-p53-treated H358 cells was greatly inhibited. In a mouse model of orthotopic human lung cancer, the tumorigenic H226Br cells, with a point mutation in p53, were inoculated intratracheally 3 days prior to the virus treatment. Intratracheal instillation of Ad5CMV-p53 prevented tumor formation in this model system suggesting that the modified adenovirus is an efficient vector for mediating transfer and expression of tumor suppressor genes in human cancer cells and that the Ad5CMV-p53 virus may be further developed into a therapeutic agent for use in cancer gene therapy.

Ad5CMV-p53 mediated a high level of expression of the p53 gene in human lung cancer cells as demonstrated by Western blot analysis. Exogenous p53 protein was approximately 14 times more abundant than the endogenous wild-type p53 in H460 cells and about two to four times more abundant than the  $\beta$ -actin internal control in H358 cells. The high level of expression may be attributed to (1) highly efficient gene transfer, (2) strong CMV promoter driving the p53 CDNA, and (3) adenoviral E1 enhancer enhancing the p53 CDNA transcription. The duration of p53 expression after infection was more than 15 days in H358 cells. However, there was a rapid decrease in expression after postinfection day 5. PCR analysis of the DNA samples from the infected H358 cells showed a decrease of the viral DNA level with the decreased protein level, indicating the loss of viral DNA during the continuous growth of cancer cells in vitro.

The decrease in p53 expression may also have resulted from cellular attenuation of the CMV promoter that controls p53 expression, since the phenomenon of host cell-mediated CMV promoter shut off has been reported previously (Dai, et al., 1992). Adenoviral vectors are nonintegrative gene transfer vectors and therefore the duration of gene expression depends upon a number of factors, including the host cells, the genes transferred, and the relevant promoter. Crystal and co-workers showed low level expression of the cystic fibrosis transmembrane conductance regulator gene in cotton rat epithelial cells was detectable 6 weeks after infection (Rosenfeld, et al., 1992). Perricaudet's laboratory demonstrated minimal expression of minidystrophin gene in mdx mouse muscle lasted for more than 3 months after infection. The short-term high level expression of the wild-type p53 protein observed in the present study may have the beneficial effect of reducing possible side effects on normal cells following in vivo treatment with Ad5CMV-p53.

The studies disclosed herein indicate that the p53 recombinant adenovirus possesses properties of tumor suppression, which appear to operate by restoring p53 protein function in tumor cells. These results provide support for the use of the Ad5CMV-p53 virion as a therapeutic agent for cancer treatment.

#### F. DNA Damaging Agents

A wide variety of DNA damaging agents may be used with the present invention, such as, agents that directly



crosslink DNA, agents that intercalate into DNA, and agents that lead to chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

Agents that directly cross-link nucleic acids, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic antineoplastic combination. Agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m<sup>2</sup> for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis, and chromosomal segregation. Examples of these compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in clinical setting for the treatment of neoplasms these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m<sup>2</sup> at 21 day intervals for adriamycin, to 35-50 mg/m<sup>2</sup> for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors, and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that these all of these factors effect a broad range of damage on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan in directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

#### G. p53 and Cisplatin Treatment

In an effort to determine the efficacy of a combination of gene replacement therapy and chemotherapy in human cancer, the inventors examined whether sequential administration of Ad-p53 and CDDP could induce apoptosis in vivo. Following 3 days of direct intratumoral injection of Ad-p53 or intraperitoneal administration of CDDP, H358 tumors implanted subcutaneously in nu/nu mice showed a

modest slowing of growth. However, if Ad-p53 and CDDP were simultaneously administered, tumors partially regressed and the tumor size remained statistically significantly smaller than those in any of the other treatment groups. The growth inhibitory effect was even more pronounced after two treatment cycles (FIG. 13A). Histologic examination revealed a massive destruction of tumor cells in the area where Ad-p53 was injected in mice treated with CDDP. In situ staining demonstrated many apoptotic cells around acellular spaces (FIGS. 13B-E). In contrast, tumors treated with CDDP alone or Ad-p53 alone showed neither acellularity nor apoptotic areas.

The present invention describes a novel strategy for human gene therapy combined with conventional chemotherapy using a DNA crosslinking agent. Tumor cell resistance to chemotherapeutic drugs represents a major problem in clinical oncology. NSCLC accounts for at least 80% of the cases of lung cancer; patients with NSCLC are, however, generally unresponsive to chemotherapy (Doyle, 1993). One goal of current cancer research is to find ways to improve the efficacy of gene replacement therapy for cancer by investigating interaction between the gene product and chemotherapeutic drugs. The herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, et al., 1992). The HS-tK gene product is an exogenous viral enzyme, whereas the wt-p53 protein is expressed in normal tissues, suggesting that the modulation of chemoresistance by alterations in wt-p53 expression might be an alternative approach using a pathway mediated by an endogenous genetic program.

An adenovirus system has potential advantages for gene delivery in vivo, such as ease of producing high titer virus, high infection efficiency, and infectivity for many types of cells. The stability and duration of expression of the introduced gene are still controversial, however. For chemogene therapy, the levels of expression and the high infectivity may be more significant than the duration of expression, because drugs can kill infected cells within several days. The increase in p53 levels in cells that are sensitive to chemotherapeutic drugs can occur within 6 hours after DNA-damaging stimuli (Fritsche, et al., 1993, Zhan, et al., 1993), although increased p53 DNA binding activity can be reversed over the course of 4 hours if the stimulus is removed (Tishler, et al., 1993). In the present model, the expression of the wt-p53 gene is driven independently by the cytomegalovirus promoter contained in an Ad-p53 vector. Therefore, a high level of p53 expression can be maintained even after cessation of drug exposure. The expression of wt-p53 protein by Ad-p53 peaks at postinfection day 3 (14-fold greater than endogenous wild type) and decreases to a low level by day 9 (Zhang, et al., 1993). This suggests that a transiently high level of wt-p53 expression is sufficient to initiate the cytotoxic program in the cancer cell.

#### H. Patients and Treatment Protocols

The inventors propose that the regional delivery of adenoviral-p53 gene constructs to lung cancer cells in patients with p53-linked cancers, such as unresectable obstructing endobronchial cancers, will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. The deliver of the p53 gene is to occur in combination with agents or factors that lead to DNA damage. This combined approach is a significant improvement on current cancer therapies, for example the loss of sensitivity to cisplatin alone, which rely on attempts to kill or remove the last cancer cell by effecting DNA

damage. As tumor cell dormancy is an established phenomenon, this makes effective killing highly unlikely.

It is anticipated that the uptake of the adenovirus constructs by NSCLC cells will decrease the rate of proliferation of these cells, however, the present examples demonstrate that the combined use of a DNA damaging agent or factor with the p53 adenovirus leads to a profound diminution of cell growth and tumor size, not shown with either factor alone. The compositions and methods disclosed herein, strongly portend an increase in the length of time the affected lung would remain expanded, prevent regrowth of the tumor and division of tumor cells, and prolong the patient's survival.

Patients with unresectable endobronchial tumor recurrence that is partially or completely obstructing the airway and that have failed or are unable to receive external beam radiotherapy will be considered for this combined protocol. Existing therapies for this condition offer only short-term palliation. Most patients have recurred despite external beam radiotherapy. It may be possible to insert a brachytherapy catheter and administer additional radiotherapy, intravenous administration of DNA damaging agents. Patients receiving current treatments have a median survival of 6 months. Patients failing brachytherapy would also be eligible to receive gene therapy. Tumor can be removed from the airway with the laser or biopsy forceps. This can be done in conjunction with injection of the adenoviral constructs thus decreasing the volume that must be injected. The administration of the viral constructs would not preclude the patient from receiving other palliative therapy if the tumor progresses.

#### I. Other Gene Transfer Techniques

Successful gene therapy generally requires the integration of a gene able to correct the genetic disorder into the host genome, where it would co-exist and replicate with the host DNA and be expressed at a level to compensate for the defective gene. Ideally, the disease would be cured by one or a few treatments, with no serious side effects. There have been several approaches to gene therapy proposed to date, which may be used with the present invention.

A first approach is to transfect DNA containing the gene of interest into cells, e.g., by permeabilizing the cell membrane either chemically or physically. This approach is generally limited to cells that can be temporarily removed from the body and can tolerate the cytotoxicity of the treatment (i.e. lymphocytes). Liposomes or protein conjugates formed with certain lipids and amphophilic peptides can be used for in vivo transfection (Stewart et al., 1992; Torchilin et al., 1992; Zhu et al., 1993), however present efficiency of gene integration is very low. It is estimated that the gene of interest integrates into the genome of only one cell in 1,000 to 100,000. In the absence of integration, expression of the transfected gene is limited to several days in proliferating cells or several weeks in non proliferating cells due to the degradation of the unintegrated DNAs.

A second approach capitalizes on the natural ability of viruses to enter cells, bringing their own genetic material with them. Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines (Miller, 1992).

A third method uses other viruses, such as adenovirus, herpes simplex viruses (HSV), cytomegalovirus (CMV), and adenoassociated virus (AAV), which are engineered to

serve as vectors for gene transfer. Although some viruses that can accept foreign genetic material are limited in the number of nucleotides they can accommodate and in the range of cells they infect, these viruses have been demonstrated to successfully effect gene expression. However, adenoviruses do not integrate their genetic material into the host genome and therefore do not require host replication for gene expression, making them ideally suited for rapid, efficient, heterologous gene expression.

Even though the invention has been described with a certain degree of particularity, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the foregoing disclosure. Accordingly, it is intended that all such alternatives, modifications, and variations which fall within the spirit and the scope of the invention be embraced by the defined claims.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

#### EXAMPLE 1

##### Construction of p53 Expression Vector

This example describes the construction of a p53 expression vector. This vector is constructed as indicated and is used to replace the E1 region (1.3–9.2 m.u.) of the Adenovirus strain Ad5 genome and employed to construct the Adenovirus virion described in Example 2.

The p53 expression cassette shown in FIG. 1, which contains human cytomegalovirus (CMV) promoter (Boshart, et al., 1985), p53 cDNA, and SV40 early polyadenylation signal, was inserted between the Xba I and Cla I sites of pXCJL1 (provided by Dr. Frank L. Graham, McMaster University, Canada).

The genome size is about 35.4 kb, divided into 100 map units (1 m.u.=0.35 kb). The p53 expression cassette replaced the E1 region (1.3–9.2 m.u.) of the Ad5 genome.

Primer 1 has the sequence 5'-GGCCCCACCCCTTGCTTC-3' (SEQ ID NO:1) and is located in the first intron downstream of the human CMV major IE gene promoter (Boshart, et al., 1985). Primer 2 has the sequence 5'-TTGTAACCAATTATAAGCTGC-3' (SEQ ID NO:2) and is located in SV40 early polyadenylation signal. Both of the primers, 15–20 bp away from the p53 cDNA insert at both ends, define a 1.40 kb PCR product. Primer 3 has the sequence 5'-TCGTTTCTCAGCAGCTGTTG-3' (SEQ ID NO:3) and primer 4 has the sequence 5'-CATCTGAACCTCAAAGCGTGG-3' (SEQ ID NO:4) and are located at 11 m.u. and 13.4 m.u. of the Ad5 genome, respectively, which define a 0.86 kb viral-genome specific PCR product.

#### EXAMPLE 2

##### Generation and Propagation of Recombinant p53 Adenovirus

This example describes one method suitable for generating helper-independent recombinant adenoviruses express-



ing p53. The molecular strategy employed to produce recombinant adenovirus is based upon the fact that, due to the packaging limit of adenovirus, pJM17 cannot form virus on its own. Therefore, homologous recombination between the p53 expression vector plasmid and pJM17 within a transfected cell results in a viable virus that can be packaged only in cells which express the necessary adenoviral proteins.

The method of this example utilizes 293 cells as host cells to propagate viruses that contain substitutions of heterologous DNA expression cassettes at the E1 or E3 regions. This process requires cotransfection of DNA into 293 cells. The transfection largely determines efficiency of viral propagation. The method used for transfection of DNA into 293 cells prior to the present invention was usually calcium-phosphate/DNA coprecipitation (Graham and van der Eb, 1973). However, this method together with the plaque assay is relatively difficult and typically results in low efficiency of viral propagation. As illustrated in this example, transfection and subsequent identification of infected cells were significantly improved by using liposomemediated transfection, when identifying the transfected cells by cytopathic effect (CPE).

The 293 cell line was maintained in Dulbecco's modified minimal essential medium supplemented with 10% heat-inactivated horse serum. The p53 expression vector and the plasmid pJM17 (McGrory, et al., 1988) for homologous recombination were cotransfected into 293 cells by DOTAP-mediated transfection according to the manufacturer's protocol (Boehringer Mannheim Biochemicals, 1992). This is schematically shown in FIG. 1.

The 293 cells (passage 35, 60% confluency) were inoculated 24 hours prior to the transfection in either 60 mm dishes or 24-well plates. The cells in each well were transfected with: 30  $\mu$ l DOTAP, 2  $\mu$ g of p53 expression vector, and 3  $\mu$ g of plasmid pJM17. After transfection cells were fed with the MEM medium every 2-3 days until the onset of CPE.

### EXAMPLE 3

#### Confirming the Identity of Recombinant Adenovirus

This example illustrates a new polymerase chain reaction (PCR) assay for confirming the identity of recombinant virions following cotransfection of the appropriate cell line.

Aliquots of cell culture supernatants (50 to 370  $\mu$ l) were collected from the test plates, treated with proteinase K (50  $\mu$ g/ml with 0.5% SDS and 20 mM EDTA) at 56° C. for 1 hour, extracted with phenol-chloroform, and the nucleic acids were ethanol precipitated. The DNA pellets were resuspended in 20  $\mu$ l dH<sub>2</sub>O and used as template for PCR amplification. The relative locations of the PCR primers and their sequences are depicted in FIG. 1 and are SEQ ID NOS:1, 2, 3 and 4, respectively. The cDNA insert-specific primers define a 1.4 kb PCR product and the viral genome-specific primers define a 0.86 kb PCR product. The PCR reactions were carried out in a 50  $\mu$ l volume containing 4 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% triton X-100, 200  $\mu$ M each of dNTPs, 10 mM Tris-Cl (pH 9.0), 2  $\mu$ M of each primer, and 1.0 unit of Taq polymerase (Promega). The reactions were carried out at 94° C., 0.5 min, 56° C., 0.5 min, and 72° C., 1 min for 30 cycles.

In order to simplify the procedure of identification of newly propagated recombinant virus, a direct PCR assay on DNA samples from cell culture supernatant was developed.

Aliquots (50 or 370  $\mu$ l) of the cell medium supernatant with CPE were treated with proteinase K and phenol/chloroform extraction. After ethanol precipitation, the DNA samples were analyzed using PCR employing two pairs of primers to amplify insert-specific and viral-genome-specific sequences. The PCR primer targets and their sequences are depicted in FIG. 1. Primers 1, 2, 3 and 4 are represented by SEQ ID NOS:1, 2, 3 and 4, respectively.

As a result, a 1.4 kb cDNA insert and a 0.86 kb viral genome fragment were amplified from the expression vector (positive control) and the DNA samples of the positive cell culture (FIG. 2B, lane 1 and 4, respectively), only the 0.86 kb fragment was amplified from the DNA sample of Ad5/RSV/GL2 virus (negative control, lane 2). No amplified bands appeared from PCR reactions that used either untreated positive cell culture medium supernatant (lane 3).

These results indicated that adenoviruses released into cell culture medium are detectable by PCR, using as little as 50  $\mu$ l of the cell culture medium supernatant for preparing DNA templates. These results will allow development of a quantitative method for using this technique to determine adenovirus titers, traditionally done by plaque assays.

The wild-type sequence of the p53 cDNA in the Ad5CMV-p53 virus was confirmed by dideoxy DNA sequencing on the CsCl-gradient-purified viral DNA. The control virus Ad5/RSV/GL2, generated in a similar manner, has a structure similar to that of Ad5CMV-p53 except a Rous sarcoma viral promoter and luciferase cDNA were used in its expression cassette. The recombinant adenovirus that carries a *E. coli*  $\beta$ -galactosidase gene (LacZ), Ad5CMV-LacZ, also has a structure similar to that of Ad5CMV-p53, and is obtainable as disclosed in Zhang et al. and from Dr. Frank L. Graham (please see Graham, et al., 1991).

Viral stock, titer, and infection. Individual clones of the Ad5CMV-p53, Ad5/RSV/GL2, and Ad5CMV-LacZ viruses were obtained by plaque-purification according to the method of Graham and Prevec (1991). Single viral clones were propagated in 293 cells. The culture medium of the 293 cells showing the completed cytopathic effect was collected and centrifuged at 1000 $\times$ g for 10 min. The pooled supernatants were aliquoted and stored at -20° C. as viral stocks. The viral titers were determined by plaque assays (Graham and Prevec, 1991). Infections of the cell lines were carried out by addition of the viral solutions (0.5 ml per 60-mm dish) to cell monolayers and incubation at room temperature for 30 min with brief agitation every 5 min. This was followed by the addition of culture medium and the return of the infected cells to the 37° C. incubator.

The gene transfer efficiency of the recombinant adenoviruses was also evaluated using Ad5CMV-LacZ in a variety of cell lines such as H226Br, H322, H460, HeLa, Hep G2, LM2, and Vero. By X-gal staining, all of the cell lines were stained 97-100% blue after infection with Ad5CMV-LacZ at an MOI of 30 PFU/cell.

### EXAMPLE 4

#### Ad5CMV-p53-Directed p53 Gene Expression in Human Lung Cancer Cells

This example describes the use of recombinant p53 adenovirus to infect human lung cancer cells with a homozygous p53 gene deletion. The results show that growth of these cells and expression of mutant p53 was suppressed, indicating the potential of the Ad5CMV-p53 virion as a useful agent for control of metastatic cells.

Immunohistochemistry was performed on infected cell monolayers that were fixed with 3.8% formalin and treated

with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 5 min. Immunohistochemical analysis was performed using Vectastain Elite kit (Vector, Burlingame, Calif.). The primary antibody used was anti-p53 antibody PAb 1801 (Oncogene Science, Manhasset, N.Y.); MOPC-21 (Organon Teknika Corp., West Chester, Pa.) was used as a negative control. The second antibody was an avidin-labeled anti-mouse IgG (Vector). The biotinylated horseradish peroxidase ABC complex reagent was used to detect the antigen-antibody complex. Finally the cells were counterstained with Harris hematoxylin (Sigma) and mounted with Cytoseal 60 (Stephens Scientific, Riverdale, N.J.).

Immunohistochemical analysis of the infected cell lines was performed to examine the *in situ* expression of p53 expression driven by the CMV promoter of the Ad5CMV-p53 virus. In the H358 cell line, which has a homozygous deletion of p53, the p53 gene was transferred with 97–100% efficiency, as detected by immunohistochemical analysis, when the cells were infected with Ad5CMV-p53 at a multiplicity of infection of 30–50 plaque-forming units (PFU)/cell (FIG. 4).

The high transfer efficiency of recombinant adenovirus was confirmed by Ad5CMV-LacZ, a virus which carries the LacZ gene transcribed by the human CMV IE promoter. At an MOI of 30–50 PFU/cell, all of the cells examined, including HeLa, Hep G2, LM2, and the human NSCLC cancer cell lines were 97–100% positive for  $\beta$ -galactosidase activity by X-gal staining. These results indicate that adenoviral vectors are an efficient vehicle for gene transfer into human cancer cells.

Western blotting analysis was performed on total cell lysates prepared by lysing monolayer cells in dishes with SDS-PAGE sample buffer (0.5 ml per 60-mm dish) after rinsing the cells with phosphate-buffered saline (PBS). For SDS-PAGE analysis lanes were loaded with cell lysates equivalent to  $5 \times 10^4$  cells (10–15 ml). The proteins in the gel were transferred to Hybond™-ECL membrane (Amersham, Arlington Heights, Ill.). The membranes were blocked with 0.5% dry milk in PBS and probed with the primary antibodies: mouse anti-human p53 monoclonal antibody PAb 1801 and mouse anti-human  $\beta$ -actin monoclonal antibody (Amersham), washed and probed with the secondary antibody: horseradish peroxidase-conjugated rabbit anti-mouse IgG (Pierce Chemical Co., Rockford, Ill.). The membranes were developed according to the Amersham's enhanced chemiluminescence protocol. Relative quantities of the exogenous p53 expressed were determined by densitometer (Molecular Dynamics Inc., Sunnyvale, Calif.).

Western blots showed the exogenous p53 protein was expressed at a high level (FIG. 5A lanes 2.3 and 5.6). The protein peaked at day 3 after infection (FIG. 6, insert, 0.5 days to 3 days). As a control, a virion with a structure similar to the recombinant Ad5CMV-p53 of Example 1 was constructed. This virion contains a luciferase cDNA driven by Rous Sarcoma Virus LTR promoter in the expression cassette of the virion. Neither p53 expression nor change in actin expression was detected in the cells infected by the virion Ad5/RSV/GL2.

The recombinant p53 adenovirus was used to infect three human lungs NSCLC cell lines: cell line H358, which has a homozygous deletion of the p53 gene, cell line H322, which has a point mutation of the p53 gene at codon 248 (G to T), and cell line H460, which has a wild-type p53 gene. The growth rate of human NSCLC cells was determined following the inoculation of H322 and H460 ( $1 \times 10^5$ ) or H358 ( $2 \times 10^5$ ) in 60-mm culture dishes 24 h before viral infection. The cells were infected with the viruses at a multiplicity of infection (MOI) of 10 PFU/cell. Culture medium was used for the mock infection control. Triplet cultures of each cell

line with different treatments were counted daily for days 1–6 after infection.

Growth of the H358 cells infected with Ad5CMV-p53 was greatly inhibited in contrast to that of noninfected cells or the cells infected with the control virion (FIG. 7A). Growth of H322 cells was also greatly inhibited by the p53 virion (FIG. 7B), while that of human lung cancer H460 cells containing wild type p53 was affected to a lesser degree (FIG. 7C). Growth of the Ad5CMV-p53 virus-infected H358 cells was inhibited 79%, whereas that of noninfected cells or the cells infected with the control virus were not inhibited. Growth of cell line H322, which has a point mutation in p53, was inhibited 72% by Ad5CMV-p53, while that of cell line H460 containing wild-type p53 was less affected (28% inhibition).

The results indicate that the p53 recombinant adenovirus possesses properties of tumor suppression, working through restoration of the p53 protein function in tumor cells.

### EXAMPLE 5

#### Ad5CMV-p53 in the Treatment of p53 Deficient Cells

The present example concerns the use of recombinant p53 adenovirus to restore growth suppression of tumor cells *in vitro* and thus to treat the malignant or metastatic growth of cells. It describes some of the ways in which the present invention is envisioned to be of use in the treatment of cancer via adenovirus-mediated gene therapy.

H358 cells were infected with Ad5CMV-p53 and Ad5/RSV/GL2 at a MOI of 10 PFU/cell. An equal amount of cells were treated with medium as a mock infection. Twenty-four hours after infection, the treated cells were harvested and rinsed twice with PBS. For each treatment, three million ( $3 \times 10^6$ ) cells in a volume of 0.1 ml were injected s.c. to each nude mouse (Harlan Co., Houston, Tex.). Five mice were used for each treatment. Mice were irradiated (300 cGy, <sup>60</sup>Co) before injection and examined weekly after injection. Tumor formation was evaluated at the end of a 6-week period and tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

To determine the inhibitory effect on tumorigenicity mediated by Ad5CMV-p53 nude mice were injected s.c. with H358 cells (a human NSCLC-type cell) to induce neoplastic growth. Each mouse received one injection of cells that had been infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 10 PFU/cell for 24 h. H358 cells treated with medium alone were used as mock-infected controls. Tumors, first palpable at postinjection day 14, were induced only by the mock- or control virus-infected cells as demonstrated in Table I:

TABLE I

Effect of Ad5CMV-p53 on tumorigenicity of H358 in nude mice\*

Treatment	No. of Tumors/ No. of Mice (%)	Mean Volume (mm <sup>3</sup> $\pm$ SD)
Medium	4/5 (80)	37 $\pm$ 12
Ad5/RSV/GL2	3/4 (75)	30 $\pm$ 14
Ad5CMV-p53	0/4 (0)	—

\*The treated H358 cells were injected s.c. at  $2 \times 10^6$  cells/mouse. Tumor sizes were determined at the end of a 6-week period.

As shown in Table I mice that received Ad5CMV-p53-treated cells did not develop tumors. The tumors at the end of a 6-week period were 4–10 mm in diameter. This study was initiated with five mice per group; one mouse each in

the Ad5CMV-p53 or Ad5/RSV/GL2 group failed to complete the study. The early deaths were presumably due to nosocomial infection.

#### EXAMPLE 6

##### Ad5CMV-p53 in the Treatment of Lung Cancer

The present example concerns the use of recombinant p53 adenovirus to restore growth suppression of tumor cells in vivo and thus to treat cancers in animals. It describes some of the ways in which the present invention is envisioned to be of use in the treatment of cancer via adenovirus-mediated gene therapy.

The efficacy of Ad5CMV-p53 in inhibiting tumorigenicity was further evaluated in the mouse model of orthotopic human lung cancer. Since H358 and H322 cells did not produce tumors in this model, cell line H226Br was used. This cell line has a squamous lung cancer origin and metastasized from lung to brain. H226Br has a point mutation (ATC to GTC) at exon 7, codon 254, of the p53 gene and is tumorigenic in mice.

The procedure for tests in the mouse model of orthotopic human lung cancer has been previously described (Georges, et al., 1993). Briefly, nude mice treated with radiation (300 cGy,  $^{60}\text{Co}$ ) were inoculated with H226Br cells by intratracheal instillation. Each mouse received  $2 \times 10^6$  cells in a volume of 0.1 ml PBS. Three days after inoculation, 10 mice per group were treated with 0.1 ml of viruses or vehicle (PBS) by intratracheal instillation once a day for two days. The virus dosage used was  $5 \times 10^7$  Ad5CMV-p53 or Ad5/RSV/GL2 per mouse. The mice were euthanized at the end of a 6-week period. Tumor formation was evaluated by dissecting the lung and mediastinum tissues and measuring the tumor size. The tumors were confirmed by histologic analysis of the sections of the tumor mass.

The irradiated nude mice were inoculated with  $2 \times 10^6$  H226Br cells/mouse by intratracheal instillation. Three days after inoculation, each of the mice (8–10 mice per group) were treated with 0.1 ml of either Ad5CMV-p53 or Ad5/RSV/GL2 or vehicle (PBS) by intratracheal instillation once a day for two days. The virus dosage used was  $5 \times 10^7$  PFU/mouse. Tumor formation was evaluated at the end of a 6-week period by dissecting the lung and mediastinum tissues and measuring the tumor size. A flow chart of the procedure is depicted in FIG. 7, with representative samples of dissection demonstrated in FIG. 8. The detected tumors were confirmed by histologic analysis. The data of tumor measurements are summarized in Table II:

TABLE II

Effect of Ad5CMV-p53 on tumorigenicity of H226Br in mouse model of orthotopic human lung cancer\*

Treatment	No. mice with Tumors/Total Mice (%)	Mean Volume (mm <sup>3</sup> ± SD)
Vehicle	7/10 (70)	30 ± 8.4
Ad5/RSV/GL2	8/10 (80)	25 ± 6.9
Ad5CMV-p53	2/8 (25)	8 ± 33 <sup>b</sup>

\*Mice were inoculated with  $2 \times 10^6$  H226Br cells/mouse intratracheally. On the 3rd day postinoculation, the mice were given either vehicle or viruses ( $5 \times 10^7$  each in 0.1 ml) intratracheally once a day for 2 days. Tumor formation was evaluated at the end of a 6-week period.

<sup>b</sup>p < 0.05 by two-way analysis of variance when compared to the groups receiving vehicle (PBS) or virus control.

Only 25% of the Ad5CMV-p53-treated mice formed tumors, whereas in the vehicle or Ad5/RSV/GL2 control

group, 70–80% of the treated mice formed tumors. The average tumor size of the Ad5CMV-p53 group was significantly smaller than those of the control groups. These results indicate that Ad5CMV-p53 can prevent H226Br from forming tumors in the mouse model of orthotopic human lung cancer.

#### EXAMPLE 7

##### Synergism between p53 and DNA Damage

The biochemical features of programmed cell death (apoptosis) show a characteristic pattern of DNA fragmentation resulting from cleavage of nuclear DNA. Recent studies have demonstrated that induction of apoptosis by chemotherapeutic drugs or ionizing radiation may be related to the status of the p53 gene and that DNA-damaging stimuli are able to elevate intracellular p53 protein levels in cells that are in the process of apoptosis (Lowe, et al., 1993, Clarke, et al., 1993, Fritsche, et al., 1993, Harper, et al., 1993, El-Deiry, et al., 1993). Inhibition of the cell cycle at the G<sub>1</sub> phase by increased levels of the wild-type p53 (wt-p53) protein allows more time for DNA repair; if optimal repair is impossible, p53 may trigger programmed cell death. Thus, p53 may contribute to the induction of apoptotic tumor cell death by chemotherapeutic agents.

Inactivation of the p53 gene by missense mutation or deletion is the most common genetic alteration in human cancers (Levine, et al., 1991, Hollstein, et al., 1991). The loss of p53 function has been reported to enhance cellular resistance to a variety of chemotherapeutic agents (Lowe, et al., 1993). The inventors studies showed that human non-small cell lung cancer (NSCLC) H358 cells, in which both alleles of p53 are deleted, were resistant to chemotherapeutic drugs, whereas cell line WTH226b, which has endogenous wt-p53, readily showed apoptotic cell death 16 hours after treatment with cisplatin (CDDP) and etoposide (VP-16) (T. Fujiwara, E. A. Grimm, T. Mukhopadhyay, J. A. Roth, unpublished data). Therefore, the inventors sought to determine whether the introduction of the wt-p53 gene into H358 cells by an adenoviral vector could increase the cell's sensitivity to the DNA crosslinking agent CDDP in vitro and in vivo.

##### Materials and Methods

H358 cells were kindly provided by A. Gazdar and J. Minna (Takahashi, et al., 1989).

##### Adenovirus Vectors

The construction and identification of a recombinant adenovirus vector that contains the CDNA that encodes human wt-p53 (Ad-p53) or luciferase (Ad-Luc) were previously reported (Zhang, et al., 1993). Briefly, the p53 expression cassette that contains human cytomegalovirus promoter, wt-p53 cDNA, and SV40 early polyadenylation signal, was inserted between the XbaI and ClaI sites of pXCJL.1. The p53 shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells (Ad5-transformed human embryonic kidney cell line) by a liposome-mediated technique. The culture supernatant of 293 cells showing the complete cytopathic effect was collected and used for subsequent infections. The control Ad-Luc virus was generated in a similar manner. Ad-p53 and Ad-Luc viruses were propagated in 293 cells. The presence of replication competent virus was excluded by HeLa cell assays. The viral titers were determined by plaque assays (Graham, et al., 1991).

##### Detection of Nucleosomal DNA Fragmentation

DNA was isolated from parental, Ad-Luc-infected, and Ad-p53-infected cells that did or did not receive CDDP

treatment, by incubating cells at 55° C. for 6 hours in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, and 50 µg/ml proteinase K). DNA was extracted twice with equal volumes of phenol and once with chloroform-isoamylalcohol (24:1) and then precipitated in ethanol. Samples were subjected to electrophoresis on a 1.5% agarose gel, and visualized by ethidium bromide staining.

TdT-mediated dUTP nick end labeling was performed according to a procedure previously reported (Gavrieli, et al., 1992). Monolayer cells were treated with 0.0% NP-40. The slides were immersed in TdT buffer (30 mM Tris-HCl, pH 7.2; 140 mM sodium cacodylate; 1 mM cobalt chloride) and incubated with biotinylated dUTP (Boehringer Mannheim, Indianapolis, Ind.) and TdT at 37° C. for 45 min. The slides were covered with 2% bovine serum albumin for 10 min and incubated with avidin-biotin complex (Vectastain Elite Kit; Vector Laboratories, Burlingame, Calif.) for 30 min. The colorimetric detection was performed by using diaminobenzidine.

#### Results

H358 cells were transduced in vitro with the human wt-p53 cDNA by exposure to Ad-p53. Western blot analysis showed a high level of wt-p53 protein expression as early as 24 hours after infection with Ad-p53, but no wt-p53 was detected in parental (uninfected) cells or control cells infected with Ad-Luc (data not shown). Concurrent immunohistochemical evaluation demonstrated detectable wt-p53 protein in more than 80% of infected cells, suggesting that the transfer and expression of p53 by Ad-p53 was highly efficient (data not shown).

Continuous exposure of Ad-p53-infected H358 cells to CDDP reduced their viability rapidly, whereas significant cell death for parental and Ad-Luc-infected cells occurred only after 72 hours of exposure to CDDP (FIG. 10A). Loss of viability was greatly enhanced in cells transduced with Ad-p53. Moreover, the reduction of viability could be observed even when cells were maintained in drug-free medium after 24 hours of exposure, suggesting that lethal damage could be induced within 24 hours (FIG. 10B). The sensitivity of wt-p53-transduced H358 cells to CDDP was dose dependent (FIG. 10C).

An internucleosomal DNA ladder indicative of DNA fragmentation was evident in cells expressing wt-p53 after 24 hours of exposure to CDDP; parental and Ad-Luc-infected cells, however, did not show DNA fragmentation (FIG. 11A). Terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine-5'-triphosphate (dUTP)-biotin nick end labeling, which detects DNA fragmentation characteristic of apoptosis in situ, showed many apoptotic cells in Ad-p53-infected cells treated with CDDP for 24 hours as shown in FIG. 11 G which demonstrates darkly staining nuclei and nuclear fragments not present in FIGS. 11B-F.

Introduction of wt-p53 is known to induce apoptosis in some types of tumor cell lines with deleted or mutated p53 (Yonish-Rouach, et al., 1991, Shaw, et al., 1992, Ramqvist, et al., 1993). However, overexpression of wt-p53 alone could not promote DNA fragmentation in the p53-negative H358 cell line (FIG. 11), although their growth was suppressed by Ad-p53 (FIG. 10). This is compatible with the inventors previous observations showing that stable H358 clones could be obtained after retrovirus-mediated wt-p53 transfer and that the clones grew more slowly than parental cells (Cai, et al., 1993).

The potential therapeutic efficacy of the combination of Ad-p53 and CDDP was evaluated in terms of the relative change in volume of H358 spheroids. The multicellular

tumor spheroid model exhibits in vitro a histologic structure similar to that of primary tumors and micrometastases. Treatment with CDDP caused a reduction of relative volume in Ad-p53-infected H358 spheroids, but had no significant effect on parental or Ad-Luc-infected spheroids (FIG. 12A). In situ TdT-mediated dUTP labeling showed many cells in the process of apoptosis on the surface of Ad-p53-infected spheroids, while no apoptotic cells were seen on spheroids not infected with Ad-p53 (FIG. 12B-E). The inventors have previously reported that retroviral-mediated wt-p53 expression inhibited growth of H322a spheroids induced by transforming growth factor  $\alpha$  (TGF- $\alpha$ ) (Fujiwara, et al., 1993). The retroviral vector could not infect H358 spheroids, however, because cells in these spheroids did not proliferate rapidly in response to exogenous TGF- $\alpha$ . The finding that exposure to CDDP reduced the size of H358 spheroids infected with Ad-p53 by inducing apoptosis on the surface suggests that Ad-p53 infects nonproliferating cells and that CDDP initiates the apoptotic process in quiescent cells.

#### EXAMPLE 8

##### Using p53 and DNA Damaging Agents in Treatment Regimens

An animal models has been employed as part of pre-clinical trials, as described hereinbelow and in Examples 5, 6 and 7. Patients for whom the medical indication for adenovirus-mediated gene transfer treatment has been established may be tested for the presence of antibodies directed against adenovirus. If antibodies are present and the patient has a history of allergy to either pharmacological or naturally occurring substances, application of a test dose of on the order of  $10^3$  to  $10^6$  recombinant adenovirus under close clinical observation would be indicated.

For the treatment of cancer using Ad5CMV-p53, recombinant adenovirus expressing p53 under the control of suitable promoter/enhancer elements, such as the CMV promoter, would be prepared and purified according to a method that would be acceptable to the Food and Drug Administration (FDA) for administration to human subjects. Such methods include, but are not limited to, cesium chloride density gradient centrifugation, followed by testing for efficacy and purity.

Two basic methods are considered to be suitable for p53 adenovirus treatment methods, a direct or local administration and a more general administration. The present methods are suitable for treating any of the variety of different cancers known to be connected with p53 mutations. In regard to general administration, a simple intravenous injection of adenovirus has been shown to be sufficient to result in viral infection of tissues at sites distant from the injection (Stratford-Perricaudet et al., 1991b), and is thus suitable for the treatment of all p53-linked malignancies. The virus may be administered to patients by means of intravenous administration in any pharmacologically acceptable solution, or as an infusion over a period of time. Generally speaking, it is believed that the effective number of functional virus particles to be administered would range from  $1 \times 10^{10}$  to  $5 \times 10^{12}$ .

Also, particularly where lung cancer is concerned, more direct physical targeting of the recombinant adenovirus could be employed if desired, in an analogous manner to the intratracheal administration of the cystic fibrosis transmembrane conductance regulator (Rosenfeld et al., 1992). This would result in the delivery of recombinant p53 adenovirus closer to the site of the target cells.

## Methods

In Situ dUTP labeling with TdT for detection of Apoptosis.

H358 spheroids were fixed on day 3 and stained as described in Example 7. Briefly, labeled TdT probes were contacted to slides immersed in TdT buffer and incubated with biotinylated dUTP and TdT at 37° C. for 45 min. The slides were covered with 2% bovine serum albumin for 10 min and incubated with avidin-biotin complex for 30 min. The calorimetric detection was performed using diaminobenzidine.

Induction of apoptosis by CDDP after in vivo infection with Ad-p53.

H358 cells ( $5 \times 10^6$ ) in 0.1 ml Hank's balanced salt solution were injected subcutaneously into the right flank of BALB/c female nu/nu mice. Thirty days later, 200  $\mu$ l of medium alone or medium containing Ad-Luc ( $10^8$  PFU/ml) or Ad-p53 ( $10^8$  PFU/ml) was injected into tumors with a diameter of 5 to 6 mm. Intratumoral injection (100  $\mu$ l) and peritumoral injection in two opposite sites (50  $\mu$ l each) were performed. CDDP (3 mg/kg) or control physiological saline was given intraperitoneally. (A) Tumor volume changes. The tumors were measured with calipers in two perpendicular diameters without the knowledge of the treatment groups, and a tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Five mice were used for each treatment group and the mean  $\pm$  SE is shown. The data was analyzed using the Student's t-test. The arrow shows the day of treatment. Two independent determinations are shown.  $p < 0.05$  from day 5 in test 1;  $p < 0.05$  from day 7 in test 2. Histologic study using the TdT-mediated biotin-dUTP labeling technique. Tumors were harvested 5 days after the beginning of treatment and immediately embedded into O. C. T. compound. Frozen tissues were cut in a cryostat at 5- $\mu$ m thicknesses. The sections were treated with 1  $\mu$ g/ml proteinase K and stained as described above. All animal care was in accordance with the UT M. D. Anderson Institutional Animal Care and Use Committee.

## Results

To demonstrate the in vivo efficacy of the methods and compositions efficacy of a combination of gene replacement therapy and chemotherapy in human cancer, the inventors examined whether sequential administration of Ad-p53 and CDDP could induce apoptosis in vivo. Following 3 days of direct intratumoral injection of Ad-p53 or intraperitoneal administration of CDDP, H358 tumors implanted subcutaneously in nu/nu mice showed a modest slowing of growth. However, if Ad-p53 and CDDP were simultaneously administered, tumors partially regressed and the tumor size remained statistically significantly smaller than those in any of the other treatment groups. The growth inhibitory effect was even more pronounced after two treatment cycles (FIG. 13A). Histologic examination revealed a massive destruction of tumor cells in the area where Ad-p53 was injected in mice treated with CDDP. In situ staining demonstrated many apoptotic cells around acellular spaces (FIG. 13B-E). In contrast, tumors treated with CDDP alone or Ad-p53 alone showed neither acellularity nor apoptotic areas.

In more detail, preferred treatment protocols may be developed along the following lines. Patients may first undergo bronchoscopy to assess the degree of obstruction. As much gross tumor as possible should be resected endoscopically. Patients should preferably undergo bronchoscopy under topical or general anesthesia. A Stifcor™ trans-bronchial aspiration needle (21 g) will be passed through the

biopsy channel of the bronchoscope. The residual tumor site would then be injected with the p53 adenovirus in a small volume such as about 10 ml or less.

In any event, since the adenovirus employed will be replication incompetent, no deleterious effect of the virus itself on subject health is anticipated. However, patients would remain hospitalized during the treatment for at least 48 hours to monitor acute and delayed adverse reactions. Safety-related concerns of the use of replication deficient adenovirus as a gene transfer vehicle in humans have been addressed in the past (Rosenfeld et al., 1992; Jaffe et al., 1992), but the dose of adenovirus to be administered should be appropriately monitored so as to further minimize the chance of untoward side effects.

There are various criteria that one should consider as presenting the existence of a need for response or the existence of toxicity. To assist in determining the existence of toxicity, the tumor bed should be photographed prior to a course of therapy. The longest diameter and its perpendicular will be measured. Size will be reported as the product of the diameters. From these data, one can calculate from these numbers the rate of regrowth of the tumor.

The time to progression can also be measured from the first observation with reduction in tumor bulk until there is evidence of progressive disease. Progressive Disease is defined as an increase of  $\geq 25\%$  in the sum of the products of the diameters of the measured lesion. Patients must have received at least two courses of therapy before a designation of progression is made. The survival of patients will be measured from entry into protocol.

Follow-up examinations would include all those routinely employed in cancer therapy, including monitoring clinical signs and taking biopsies for standard and molecular biological analysis in which the pattern of expression of various p53 genes could be assessed. This would also supply information about the number of cells that have taken up the transferred gene and about the relative promoter strength in vivo. Based on the data obtained adjustments to the treatment may be desirable. These adjustments might include adenovirus constructs that use different promoters or a change in the number of pfu injected to ensure a infection of more, or all, tumor cells without unphysiological overexpression of the recombinant genes.

It is contemplated that the expression of exogenous genes transferred in vivo by adenovirus can persist for extended periods of time. Therapeutically effective long-term expression of virally transferred exogenous genes will have to be addressed on a case by case basis. Marker genes are limited in their usefulness to assess therapeutically relevant persistence of gene expression as the expression levels required for the amelioration of any given genetic disorder might differ considerably from the level required to completely cure another disease.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. All claimed matter and methods can be made and executed without undue experimentation.

## REFERENCES

The following references to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

- Bargonetti, et al. (1991) *Cell* 65:1083-1091.  
 Bishop (1987) *Science* 235:305-311.  
 Boehringer Mannheim Biochemicals (1992). DOTAP for high efficiency transfections. *BMBiochemica* 9(1):17.  
 Boshart, M. et al. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell*, 41:521-530.  
 Cai, D. W., Mukhopadhyay, T., Liu, T., Fujiwara, T., and Roth, J. A. Stable expression of the wild-type p53 gene in human lung cancer cells after retrovirus-mediated gene transfer. *Human Gene Ther*, 4:617-624, 1993.  
 Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science*, 256:1550-1552, 1992.  
 Casey, G. Lo-Hueh, M., Lopez, M. E., Vogelstein, B., and Stanbridge, E. J. (1991). Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene. *Oncogene* 6:1791-1797.  
 Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, 362: 849-852, 1993.  
 Dai, et al. (1992) *Proc. Natl. Acad. Sci.* 89:10892-10895.  
 Doyle, L. A. Mechanisms of drug resistance in human lung cancer cells. *Semin Oncol*, 20:326-337, 1993.  
 El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell*, 75:817-825, 1993.  
 Fritsche, M., Haessler, C., and Brandner, G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene*, 8:307-318, 1993.  
 Fields et al. (1990) *Science* 249:1046-1049.  
 Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Cai, D. W., Owen-Schaub, L. B., and Roth, J. A. A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res*, 53:4129-4133, 1993.  
 Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. *J Cell Biol*, 119:493-501, 1992.  
 Georges et al. (1993) *Cancer Res* 53:1743-1746.  
 Ghosh-Choudhury and Graham (1987) *Biochem. Biophys. Res. Comm.* 147:964-973.  
 Gluzman et al., (1982) in *Eukaryotic Viral Vectors* (Gluzman, Y., Ed.) pp. 187-192, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.  
 Graham, F. L. and A. J. van der Eb. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.  
 Graham, F. L. and Prevec, L. Manipulation of adenovirus vectors.  
 In: Murray E. J. (ed.), *Methods in Molecular Biology, Gene Transfer and Expression Protocols*, pp. 109-128. New Jersey: The Humana Press Inc. 1991.  
 Graham, F. L., J. Smiley, W. C. Russell and R. Nairn (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen Virol*. 36:59-72.  
 Grunhaus, A. and Horwitz, M. S. (1992). Adenoviruses as cloning vectors. *Semin. Virology* 3:237-2542.  
 Harper, J. E., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, 75:805-816, 1993.  
 Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. (1991). p53 mutations in human cancers. *Science* 253:49-53.  
 Jaffe et al., (1992) *Nature Genetics* 1:372-378.  
 Le Gal et al., (1993) *Science* 259:988-990.  
 Ledley, J. (1987). *J. Pediatrics* 110, 1.  
 Levine, A. J., Momand, J., and Finlay, C. A. The p53 tumour suppressor gene. *Nature*, 351:453-456, 1991.  
 Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, 74:957-967, 1993.  
 Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, 362:847-849, 1993.  
 McGrory, W. J. et al. (1988). A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 163:614-617.  
 Mercer, W. E. (1992). Cell cycle regulation and the p53 tumor suppressor protein. *Critic. Rev. Eukar. Gene Express.* 2:251-263.  
 Mietz, et al. (1992) *EMBO* 11:5013-5020.  
 Miller 1992. *Curr. Top. Microbiol. Immunol.* 158:1  
 Montenarh, M. (1992). Biochemical, immunological, and functional aspects of the growth-suppressor/oncoprotein p53. *Critic. Rev. Onco.* 3:233-256.  
 Mulligan, (1993). *Science* 260:926.  
 Nicolau, C., et al. (1983). *Proc. Natl. Acad. Sci. U.S.A.* 80, 1068.  
 Ragot et al., (1993) *Nature*, 361:647-650.  
 Ramqvist, T., Magnusson, K. P., Wang, Y., Szekeley, L., and Klein, G. Wild-type p53 induces apoptosis in a Burkitt lymphoma (BL) line that carries mutant p53. *Oncogene*, 8: 1495-1500, 1993.  
 Rosenfeld et al., (1992) *Cell* 68:143-155.  
 Rosenfeld et al., (1991) *Science*, 232:431-434.  
 Roth, J., Ruckdeschel, Weisenburger, Editors, *Thoracic Oncology*. First Edition. Chapter 49, pgs 711-721. Sanders, N.Y., 1989.  
 Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. Induction of apoptosis by wild-type 53 in a human colon tumor-derived cell line. *Proc Natl Acad Sci USA*, 89: 4495-4499, 1992.  
 Spandidos, et al. (1989). *J. Pathol.*, 157:1-10.  
 Stewart et al., 1992, *Hum. Gene Ther.* 3:267  
 Stratford-Perricaudet, L. and M. Perricaudet. (1991a). Gene transfer into animals: the promise of adenovirus. p. 51-61. In O. Cohen-Haguenaier and M. Boiron (Eds.), *Human Gene Transfer*. Editions John Libbey Eurotext, France.  
 Stratford-Perricaudet et al., (1991b) *Hum. Gene. Ther.* 1:241-256  
 Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. *Science*, 246:491-494, 1989.  
 Takahashi, T., Carbone, D., Takahashi, T., Nau, M. M., Hida, T., Linnoila, I., Ueda, R., and Minna, J. D. (1992). Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. 1992. *Cancer Res*. 52:2340-2342.  
 Tishler, R. B., Calderwood, S. K., Coleman, C. N., and Price, B. D. Increases in sequence specific DNA binding by p53

- following treatment with chemotherapeutic and DNA damaging agents. *Cancer Res*, 53:2212-2216, 1993.
- Tooza, J. (1981). *Molecular biology of DNA Tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
- Torchilin et al., 1992, *Faseb J.* 6:2716
- Travali, et al. (1990). *FASEB*, 4:3209-3214.
- Weinberg, R. A. (1991). *Tumor suppressor gene. Science* 254:1138-1145.
- Wilcock, et al. (1991) *Nature* 349:429-431.
- Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990) Direct gene transfer into mouse muscle in vivo. *Science* 247:1465-1468.
- Yonish-Rouach, E., Resnitzky, D., Rotem, J., Sachs, L., Kimchi, A., and Oren, M. Wild-type p53 induces apop-

- toxis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature*, 352:345-347, 1991.
- Zakut-Houri et al. (1985). *EMBO J.*, 4:1251-1255.
- Zhan, Q., Carrier, F. and Fornace Jr., A. J. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol Cell Biol*, 13: 4242-4250, 1993.
- Zhang, W. W., Fang, X., Branch, C. D., Mazur, W., French, B. A., and Roth, J. A. Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. *BioTechniques*, 1993.
- 10 Zhang, W. W., Fang, X., Mazur, W., French, B. A., Georges, R. N., and Roth, J. A. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Therapy*, 1993.
- Zhu, et al., 1993, *Science* 261:209-211.

## SEQUENCE LISTING

## ( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 4

## ( 2 ) INFORMATION FOR SEQ ID NO:1:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 19 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCCCCACCCC CTTGCTTTC

19

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTGTAAACCAT TATAAGCTGC

20

## ( 2 ) INFORMATION FOR SEQ ID NO:3:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCGTTTCTCA GCAAGCTGTTG

20

## ( 2 ) INFORMATION FOR SEQ ID NO:4:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear



-continued

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATCTGAACT CAAAGCGTGG

20

What is claimed is:

1. A method of killing a tumor cell in a patient in need thereof, comprising directly administering to said tumor cell therapeutically effective amounts of a viral vector and a DNA damaging agent, wherein said viral vector comprises a DNA sequec encoding p53 operatively linked to a promoter, and wherein expression of said p53 and DNA damage result in the killing of said tumor cell.

2. The method of claim 1, wherein said viral vector is selected from the group consisting of retrovirus, adenovirus, herpesvirus, adeno-associated virus and cytomegalovirus.

3. The method claim 2, wherein the tumor cell is contacted with a pharmaceutical composition comprising a DNA damaging compound.

4. The method of claim 3, wherein the DNA damaging agent is cisplatin.

5. The method of claim 4, wherein said cisplatin is administered at 20 mg/m<sup>2</sup>.

6. The method of claim 3, wherein the DNA damaging agent is doxorubicin.

7. The method of claim 6, wherein said doxorubicin is administered at 25-75 mg/m<sup>2</sup>.

8. The method of claim 3, wherein the DNA damaging agent is etoposide.

9. The method of claim 8, wherein said etoposide is administered at 35-50 mg/m<sup>2</sup>.

10. The method of claim 3, wherein the DNA damaging agent is verapamil.

11. The method of claim 3, wherein the DNA damaging agent is podophyllotoxin.

12. The method of claim 3, wherein the DNA damaging agent is 5-FU.

13. The method of claim 12, wherein said 5-FU is administered at 3-15 mg/kg.

14. The method of claim 2, wherein said viral vector is a retroviral vector.

15. The method of claim 2, wherein said viral vector is an adenoviral vector.

16. The method of claim 15, wherein the amount of adenoviral vector is  $1 \times 10^5$  to  $1 \times 10^{12}$  pfu.

17. The method of claim 16, wherein said amount is  $5 \times 10^7$  pfu.

18. The method of claim 16, wherein said amount is  $2 \times 10^7$  pfu.

19. The method of claim 2, wherein said viral vector is a herpesviral vector.

20. The method of claim 2, wherein said viral vector is an adeno-associated viral vector.

21. The method of claim 2, wherein said viral vector is a cytomegaloviral vector.

22. The method of claim 1, wherein said promoter is a constitutives promoter.

23. The method of claim 22, wherein the promoter is selected from the group consisting of SV40, CMV and RSV.

24. The method of claim 23, wherein the promoter is the CMV IE promoter.

25. The method of claim 24, wherein the viral vector further comprises a polyadenylation signal.

26. The method of claim 25, wherein the viral vector is an adenoviral vector.

27. The method of claim 1, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with X-ray radiation, UV-irradiation,  $\gamma$ -irradiation or microwaves.

28. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with X-ray radiation.

29. The method of claim 28, wherein the x-ray dosage is between 2000 and 6000 roentgens.

30. The method of claim 28, wherein the x-ray -dosage is between 50 and 200 roentgens.

31. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with UV-irradiation.

32. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with  $\gamma$ -irradiation.

33. The method of claim 27, wherein the tumor cell is contacted with a DNA damaging agent by irradiating the tumor cell with microwaves.

34. The method claim 1, wherein the tumor cell is contacted with a DNA damaging agent by administering to the patient a pharmaceutical composition comprising a DNA damaging compound.

35. The method of claim 1, wherein said viral vector is administered prior to said DNA damaging agent.

36. The method of claim 1, wherein said viral vector is administered after said DNA damaging agent.

37. The method of claim 1, wherein said viral vector is administered at the same time as said DNA damaging agent.

38. The method of claim 1, wherein said viral vector is delivered endoscopically, intravenously, intratracheally, intralesionally, percutaneously or subcutaneously.

39. The method of claim 1, wherein said tumor is located in a resected tumor bed.

40. The method of claim 1, wherein said administering is repeated.

41. The method of claim 1, wherein said tumor cell is a lung cancer cell.

42. The method of claim 41, wherein said lung cancer cell is non-small cell lung carcinoma cell.

43. The method of claim 42, wherein said non-small cell lung carcinoma cell is a squamous carcinoma cell.

44. The method of claim 42, wherein said non-small cell lung carcinoma cell is an adenocarcinoma cell.

45. The method of claim 42, wherein said non-small cell lung carcinoma cell is a large-cell undifferentiated carcinoma cell.

46. The method of claim 41, wherein said lung cancer cell is a small cell lung carcinoma cell.

47. The method of claim 1, wherein said tumor cell is an epithelial tumor cell.

48. The method of claim 1, wherein said tumor cell is a breast cancer cell.

49. The method of claim 1, wherein said viral vector is administered in about 0.1 ml.



50. The method of claim 1, wherein said viral vector is administered in about 10 ml.

51. A method of treating cancer in a cancer patient, comprising directly administering to a tumor site therapeutically effective amounts of a viral vector and a DNA damaging agent, wherein said viral vector comprises a DNA sequence encoding p53 operatively linked to a promoter, and wherein expression of said p53 and DNA damage result in treatment of said cancer.

52. The method of claim 51, wherein said viral vector is selected from the group consisting of retrovirus, adenovirus, herpesvirus, adeno-associated virus and cytomegalovirus.

53. The method of claim 52, wherein said viral vector is a retroviral vector.

54. The method of claim 52, wherein said viral vector is an adenoviral vector.

55. The method of claim 54, wherein the amount of adenoviral vector is  $1 \times 10^5$  to  $1 \times 10^{12}$  pfu.

56. The method of claim 55, wherein said amount is  $5 \times 10^7$  pfu.

57. The method of claim 55, wherein said amount is  $2 \times 10^7$  pfu.

58. The method of claim 52, wherein said viral vector is a herpesviral vector.

59. The method of claim 52, wherein said viral vector is an adeno-associated viral vector.

60. The method of claim 52, wherein said viral vector is a cytomegaloviral vector.

61. The method of claim 51, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with X-ray radiation, UV-irradiation,  $\gamma$ -irradiation or microwaves.

62. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with X-ray radiation.

63. The method of claim 62, wherein the x-ray dosage is between 2000 and 6000 roentgens.

64. The method of claim 62, wherein the x-ray dosage is between 50 and 200 roentgens.

65. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with UV-irradiation.

66. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with  $\gamma$ -irradiation.

67. The method of claim 61, wherein the tumor site is contacted with a DNA damaging agent by irradiating the tumor site with microwaves.

68. The method claim 51, wherein the tumor site is contacted with a DNA damaging agent by administering to the patient a pharmaceutical composition comprising a DNA damaging compound.

69. The method of claim 68, wherein the DNA damaging compound is cisplatin.

70. The method of claim 69, wherein said cisplatin is administered at 20 mg/m<sup>2</sup>.

71. The method of claim 68, wherein the DNA damaging agent is doxorubicin.

72. The method of claim 71, wherein said etoposide is administered at 35-50 mg/m<sup>2</sup>.

73. The method of claim 72, wherein said doxorubicin is administered at 25-75mg/m<sup>2</sup>.

74. The method of claim 68, wherein the DNA damaging agent is etoposide.

75. The method of claim 68, wherein the DNA damaging agent is verapamil.

76. The method of claim 68, wherein the DNA damaging agent is podophyllotoxin.

77. The method of claim 68, wherein the DNA damaging agent is 5-FU.

78. The method of claim 77, wherein said 5-FU is administered at 3-15 mg/kg.

79. The method of claim 51, wherein said viral vector is administered prior to said DNA damaging agent.

80. The method of claim 79, wherein the period between administration of the viral vector and DNA damaging agent is between 12 and 24 hours.

81. The method of claim 79, wherein the period between administration of the viral vector and DNA damaging agent is between 6 and 12 hours.

82. The method of claim 79, wherein the period between administration of the viral vector and DNA damaging agent is about 12 hours.

83. The method of claim 51, wherein said viral vector is administered after said DNA damaging agent.

84. The method of claim 83, wherein the period between administration of the DNA damaging agent and viral vector is between 12 and 24 hours.

85. The method of claim 83, wherein the period between administration of the DNA damaging agent and viral vector is between 6 and 12 hours.

86. The method of claim 83, wherein the period between administration of the DNA damaging agent and viral vector is about 12 hours.

87. The method of claim 51, wherein said viral vector is administered at the same time as said DNA damaging agent.

88. The method of claim 51, wherein said viral vector is delivered endoscopically, intravenously, intratracheally, intralesionally, percutaneously or subcutaneously.

89. The method of claim 51, wherein said tumor site is a resected tumor bed.

90. The method of claim 51, wherein said administration is repeated.

91. The method of claim 51, wherein said cancer is a lung cancer.

92. The method of claim 91, wherein said lung cancer is a non-small cell lung carcinoma cancer.

93. The method of claim 92, wherein said non-small cell lung carcinoma cancer is a squamous carcinoma cancer.

94. The method of claim 92, wherein said non-small cell lung carcinoma cancer is an adenocarcinoma cancer.

95. The method of claim 92, wherein said non-small cell lung carcinoma cancer is a large-cell undifferentiated carcinoma cancer.

96. The method of claim 91, wherein said lung cancer is a small cell lung carcinoma cancer.

97. The method of claim 51, wherein said cancer is an epithelial cancer.

98. The method of claim 51, wherein said cancer is breast cancer.

99. The method of claim 51, wherein said viral vector is administered in about 0.1 ml.

100. The method of claim 51, wherein said viral vector is administered in about 10 ml.

101. The method of claim 2, wherein said promoter is a constitutives promoter.

102. The method of claim 101, wherein said promoter is selected from the group consisting of SV40, CMV and RSV.

103. The method of claim 102, wherein the promoter is the CMV IE promoter.

104. The method of claim 103, wherein the viral vector further comprises a polyadenylation signal.

105. The method of claim 104, wherein the viral vector is an adenoviral vector.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 5,747,469  
DATED : May 5, 1998  
INVENTOR(S) : Roth et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Page 3, item [56], insert --Petty et al., "Expression of the p53 tumour suppressor gene product is a determinant of chemosensitivity," Journal of Cancer Research and Clinical Oncology, 120:R108, 1994.--

In claim 1, column 37, line 15, delete "sequec" and insert --sequence-- therefor.

In claim 7, column 37, line 30, delete "doxorabycin" and insert --doxorubicin-- therefor.

In claim 22, column 37, line 61, delete "constitutives" and insert --constitutive-- therefor.

In claim 30, column 38, line 21, delete "-dosage" and insert --dosage-- therefor.

In claim 43, column 38, line 54, delete "sqamous" and insert --squamous-- therefor.

In claim 68, column 39, line 50, delete "a a" and insert --a-- therefor.

In claim 73, column 39, line 61, delete "25-75mg/m<sup>2</sup>" and insert --25-75 mg/m<sup>2</sup>-- therefor.

In claim 85, column 40, line 23, delete "12hours" and insert --12 hours-- therefor.

In claim 101, column 40, line 57, delete "2" and insert --51-- therefor.

In claim 101, column 40, line 58, delete "constitutives" and insert --constitutive-- therefor.

Signed and Sealed this

Twenty-seventh Day of June, 2000

Attest:



Q. TODD DICKINSON

Attesting Officer

Director of Patents and Trademarks



US006017524A

**United States Patent** [19][11] **Patent Number:** **6,017,524****Roth et al.**[45] **Date of Patent:** **Jan. 25, 2000**[54] **INHIBITING THE GROWTH P53 DEFICIENT TUMOR CELLS BY ADMINISTERING THE P53 GENE**[75] **Inventors:** Jack A. Roth; Tapas Mukhopadhyay; Michael A. Tainsky, all of Houston, Tex.[73] **Assignee:** Board of Regents, The University of Texas System, Austin, Tex.[21] **Appl. No.:** 07/960,513[22] **Filed:** Oct. 13, 1992**Related U.S. Application Data**

[63] Continuation-in-part of application No. 07/665,538, Mar. 6, 1991, abandoned.

[51] **Int. Cl.<sup>7</sup>** ..... A61K 48/00; C12N 15/00[52] **U.S. Cl.** ..... 424/93.2; 514/44; 435/172.3; 435/320.1; 935/62[58] **Field of Search** ..... 435/320.1, 172.3; 514/44; 424/93 B, 93.2; 935/62[56] **References Cited****U.S. PATENT DOCUMENTS**

4,740,463 4/1988 Weinberg et al. .... 435/172.3  
 4,980,289 12/1990 Temin et al. .  
 5,055,400 10/1991 Lo et al. .

**FOREIGN PATENT DOCUMENTS**

0351585 6/1989 European Pat. Off. .  
 0 390 323 10/1990 European Pat. Off. .... C12Q 1/68  
 0475623 8/1991 European Pat. Off. .  
 0174608 9/1995 European Pat. Off. .  
 2688514 9/1993 France .  
 WO 90/10448 9/1990 WIPO .  
 WO 93/03769 3/1993 WIPO .  
 WO 94/10323 5/1994 WIPO .  
 WO 94/24297 10/1994 WIPO .  
 WO 95/02697 1/1995 WIPO .

**OTHER PUBLICATIONS**

Casey et al., "Growth Suppression of Human Breast Cancer Cells by the Introduction of a Wild-Type p53 Gene," *Oncogene*, 6:1791-1797, 1991.

Wills and Menzel, "Adenovirus Vectors for Gene Therapy of Cancer," *Journal of Cellular Biochemistry*, p. 204, Abstract # S216, Mar.-Apr. 1993.

Zhang et al., "Generation and Identification of Recombinant Adenovirus by Liposome-Mediated Transfection and PCR Analysis," *BioTechniques*, 15(5):868-872, 1993.

Bandyopadhyay & Temin, "Expression of Complete Chicken Thymidine Kinase Gene Inserted in a Tetrovirus Vector," *Molecular and Cellular Biology*, 4(4):749-754, 1984.

Bowtell et al., "Comparison of Expression in Hemopoietic Cells by Retroviral Vectors Carrying Two Genes," *Journal of Virology*, 62(7):2464-2473, 1988.

Casson et al., "p53 Gene Mutations in Barrett's Epithelium and Esophageal Cancer," *Cancer Research*, 51:4495-4499, 1991.

Chen et al., "Genetic Mechanisms of Tumor Suppression by the Human p53 Gene," *Science*, 250:1576-1580, 1990.

Chen et al., "Expression of Wild-Type p53 in Human A673 Cells Suppresses Tumorigenicity but Not Growth Rate," *Oncogene*, 6:1799-1805, 1991.

Goyette et al., "Progression of Colorectal Cancer Is Associated with Multiple Tumor Suppressor Gene Defects but Inhibition of Tumorigenicity Is Accomplished by Correction of Any Single Defect via Chromosome Transfer," *Molecular and Cellular Biology*, 12(3):1387-1395, 1992.

Gusterson et al., "Expression of p53 in Premalignant and Malignant Squamous Epithelium," *Oncogene*, 6:1785-1798, 1991.

Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science*, 248:1101-1104, 1990.

Maxwell et al., "Inefficiency of Expression of Luciferase Reporter from Transfected Murine Leukaemia Proviral DNA May Be Partially Overcome by Providing a Strong Polyadenylation Signal," *Journal of General Virology*, 72:1721-1724, 1991.

Mukhopadhyay et al., "Specific Inhibition of K-ras Expression and Tumorigenicity of Lung Cancer Cells by Antisense RNA," *Cancer Research*, 51:1744-1748, 1991.

Owens & Boyd, "Expressing Antisense Po RNA in Schwann Cells Perturbs Myelination," *Development*, 112:639-649, 1991.

Palmer et al., "Efficient Retrovirus-Mediated Transfer and Expression of a Human Adenosine Deaminase Gene in Diploid Skin Fibroblasts from an Adenosine Deaminase-Deficient Human," *Proceedings of the National Academy of Science USA*, 84:1055-1059, 1987.

(List continued on next page.)

*Primary Examiner*—Deborah Crouch  
*Attorney, Agent, or Firm*—Arnold, White & Durkee

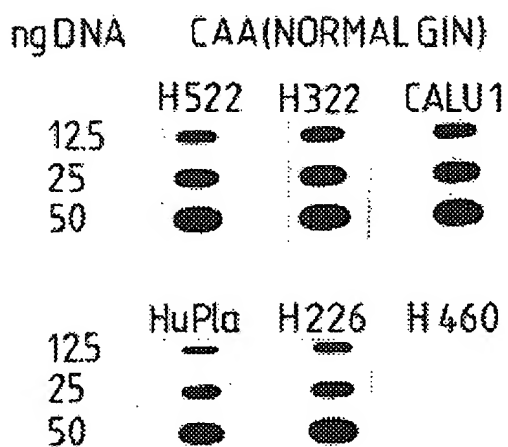
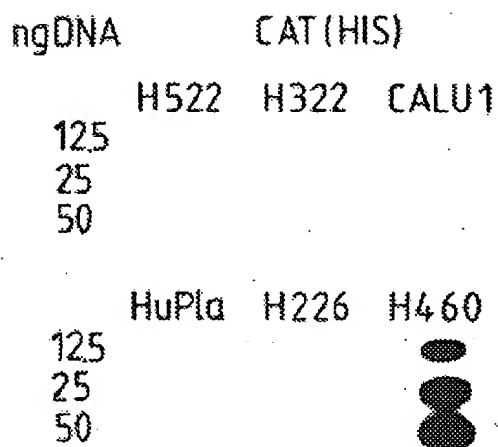
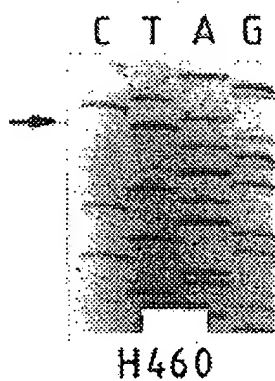
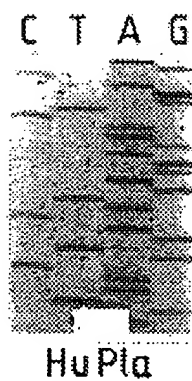
[57] **ABSTRACT**

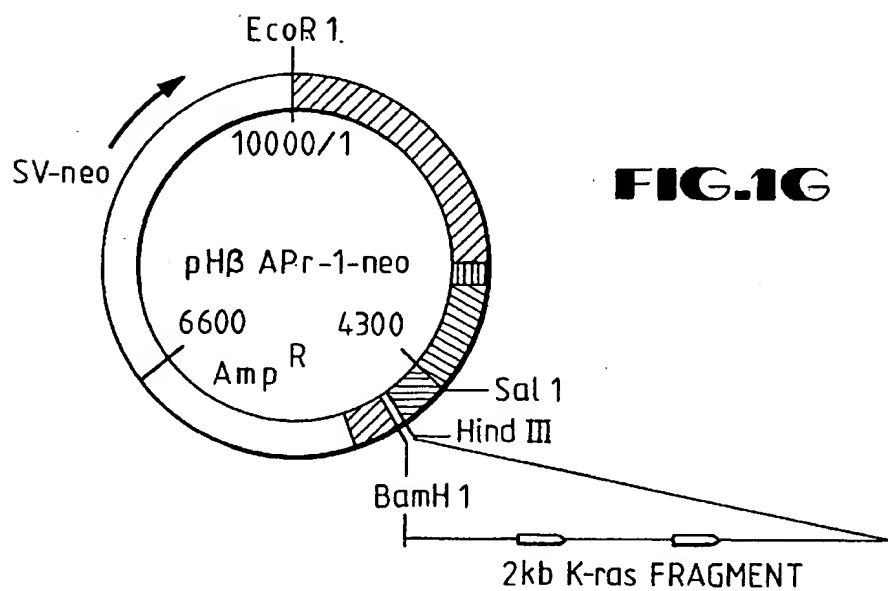
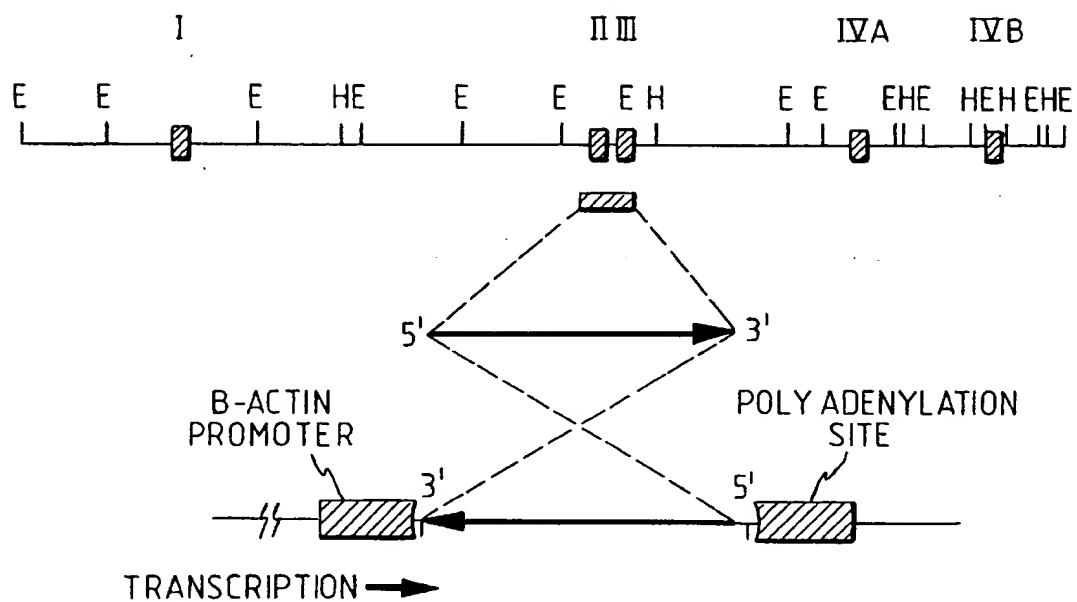
Disclosed are methods and compositions for the selective manipulation of gene expression through the preparation of retroviral expression vectors for expressing antisense sequences, such as K-ras oncogene antisense sequences, or sequences encoding a desired product, such as wild type p53 sequences. Preferred retroviral vectors of the present invention incorporate the  $\beta$ -actin promoter in a reverse orientation with respect to retroviral transcription. Preferred antisense RNA constructs of the present invention employ the use of antisense intron DNA corresponding to distinct intron regions of the gene whose expression is targeted for down-regulation. In an exemplary embodiment, a human lung cancer cell line (NCI-H460a) with a homozygous spontaneous K-ras mutation was transfected with a recombinant plasmid that synthesizes a genomic segment of K-ras in antisense orientation. Translation of the mutated K-ras mRNA was specifically inhibited, whereas expression of H-ras and N-ras was unchanged. A three-fold growth inhibition occurred in H460a cells when expression of the mutated ras p21 protein was down-regulated by antisense RNA and cells remained viable. The growth of H460a tumors in nu/nu mice was substantially reduced by expressed K-ras antisense RNA.

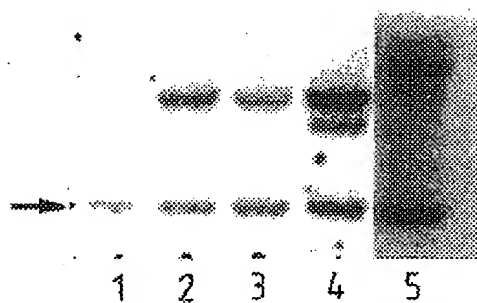
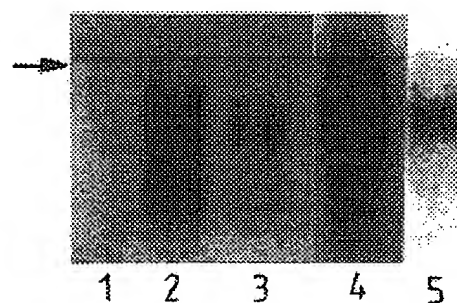
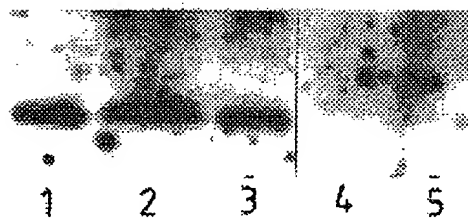
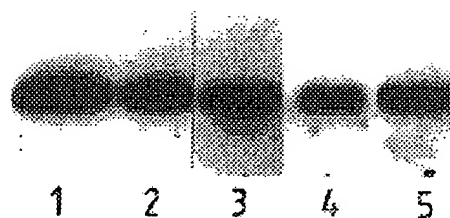
**41 Claims, 15 Drawing Sheets**

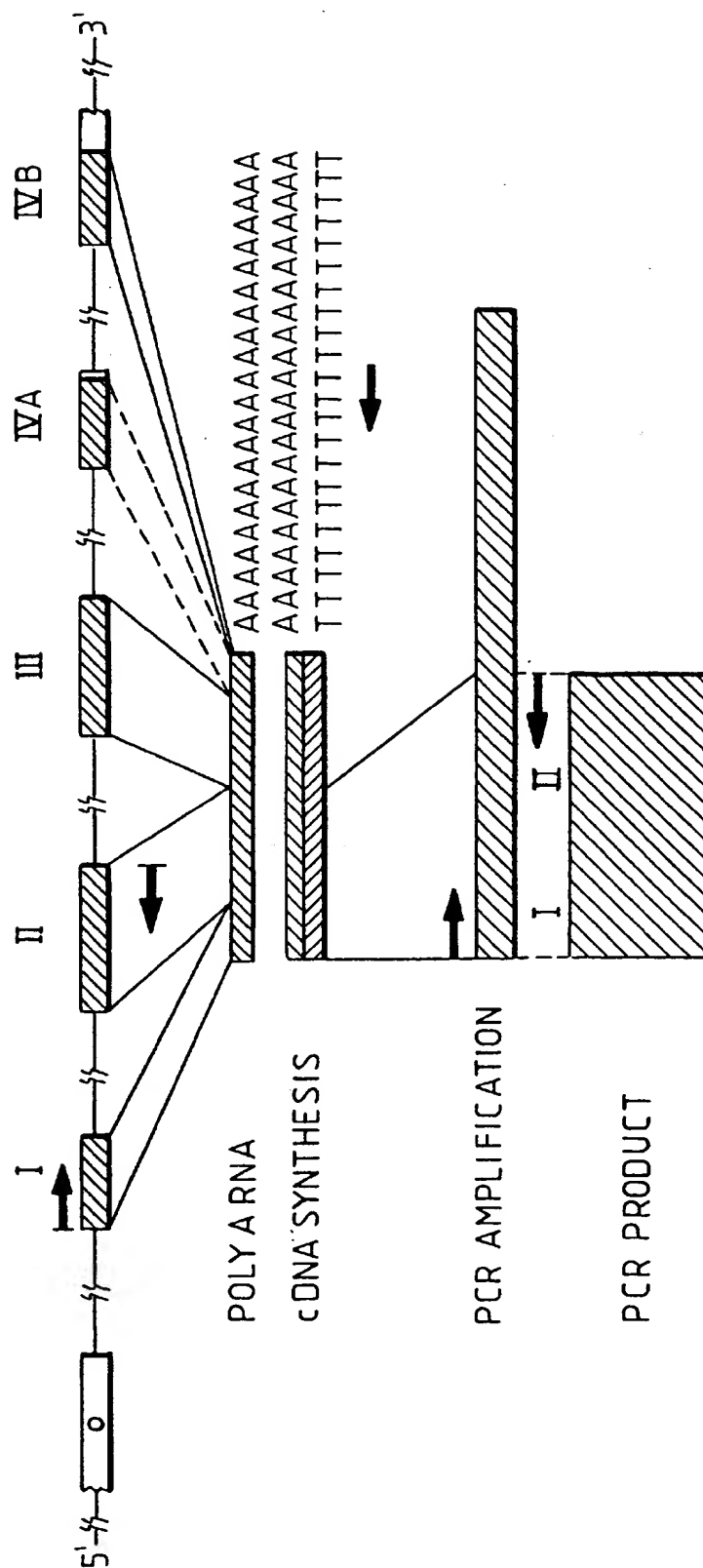
## OTHER PUBLICATIONS

- Seyama et al., "In Vitro and In Vivo Regulation of Liver Epithelial Cells Carrying a Metallothionein-rasT24 Fusion Gene," *Molecular Carcinogenesis*, 1:89-95, 1988.
- Takahashi et al., "Wild-Type but Not Mutant p53 Suppresses the Growth of Human Lung Cancer Cells Bearing Multiple Genetic Lesions," *Cancer Research*, 52:2340-2343, 1992.
- Zhou & Duesberg, "myc Protooncogene Linked to Retroviral Promoter, but Not to Enhancer, Transforms Embryo Cells," *Proceedings of the National Academy of Science USA*, 85:2924-2928, 1988.
- Conroy, "New Gene Therapy Cleared for Use Against Lung Cancer," *Biotech Daily*, pp. 3-4, Sep. 18, 1992.
- Sundaresan, V., et al., "Somatic Genetic Changes in Pre-Invasive Lesions in Bronchial Epithelium," *J. Pathol.*, 167(Suppl) 1992, 100A, Abstract Only.
- Dialog Search Reports dated Aug. 7, 1992 and Feb. 26, 1993.
- Kasid et al (1989) *Science* 243, 1354-1356.
- Debus et al., *J Cancer Res Clin Oncol*, 116(Suppl Part 1):5-162, Abstract # A2.037.09, 1990.
- Delauney et al., "A stable bifunctional antisense transcript inhibiting gene expression in transgenic plants," *Proc. Natl. Acad. Sci. USA*, 85:4300-4304, 1988.
- Feig, et al., "Somatic Activation of ras<sup>K</sup> Gene in a Human Ovarian Carcinoma," *Science*, 223:698-701, 1984.
- Finkel, et al., "Activation of ras Genes in Human Tumors Does Not Affect Localization, Modification, or Nucleotide Binding Properties of p21," *Cell*, 37:151-158, 1984.
- Griep and Heiner, "Antisense Myc sequences induce differentiation of F9 cells," *Proc. Natl. Acad. Sci. USA*, 85:6806-6810, 1988.
- Gunning, et al., "A human  $\beta$ -actin expression vector system directs high-level accumulation of antisense transcripts," *Proc. Natl. Acad. Sci. USA*, 84:4831-4835, 1987.
- Kasid, et al., "Effect of Antisense c-raf-1 on Tumorigenicity and Radiation Sensitivity of a Human Squamous Carcinoma," *Science*, 243:1354-1356, 1989.
- Khokha, Rama, et al., "Antisense RNA-Induced Reduction in Murine TIMP Levels Confers Oncogenicity on Swiss 3T3 Cells," *Science*, 243:947-950, 1989.
- Kris, et al., "Expression of Ki-Ras Oncogene in Tumor Cell Variants Exhibiting Different Metastatic Capabilities," *Int. J. Cancer*, 35:227-230, 1985.
- Izant and Weintraub, "Inhibition of Thymidine Kinase Gene Expression by Anti-Sense RNA: A Molecular Approach to Genetic Analysis," *Cells*, 36:1007-1015, 1984.
- Johnson, et al., "Transfection of a Rat Cell Line with the v-Ki-ras Oncogene is Associated with Enhanced Susceptibility to Natural Killer Cell Lysis," *J. Exp. Med.*, 162:1732-1737, 1985.
- McGrath, et al., "Structure and organization of the human Ki-ras proto-oncogene and a related processed pseudogene," *Nature*, 304:501, 1983.
- Magrath, "Tumor-specific antisense oligonucleotides for controlling cancer", Abstract No. 114:55778n, *Chemical Abstracts*, 114(7):68 (1991).
- Mercola, et al., "Antisense RNA: Eukaryotic Controls", *Gene*, 72:253-265 (1988).
- Miller and Rosman, Improved Retroviral Vectors for Gene Transfer and Expression, *BioTechniques*, 7(9):980-990, 1989.
- Munroe, Stephen H., "Antisense RNA inhibits splicing of pre-mRNA in vitro", *The EMBO Journal*, 7(8):2523-2532 (1988).
- Prochownik, et al., "c-myc Antisense Transcripts Accelerate Differentiation and Inhibit G<sub>1</sub> Progression in Murine Erythroleukemia Cells", *Molecular and Cellular Biology*, 8(9):3683-3695, 1988.
- Santos, et al., Malignant Activation of a K-ras Oncogene in Lung Carcinoma but Not in Normal Tissue of the Same Patient, *Science*, 223:661-664, 1984.
- Shimizu, et al., "Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1", *Nature*, 304:497-500, 1983.
- Stowers, et al., "Activation of the K-ras Protooncogene in Lung Tumors from Rats and Mice Chronically Exposed to Tetranitromethane", *Cancer Research*, 47:3212-3219, 1987.
- Taya, et al., "A novel combination of K-ras and myc amplification accompanied by point mutational activation of K-ras in a human lung cancer", *The EMBO Journal*, 3(12):2943-2946, 1984.
- Toftgard, et al., "Proto-oncogene expression during two-stage carcinogenesis in mouse skin", *Carcinogenesis*, 6(4):655-657, 1985.
- Vogelstein, et al., "Genetic Alterations During Colorectal-Tumor Development", *The New England Journal of Medicine*, 319(9):525-532, 1988.
- Wahran et al., *Tumour Biol*, 6:41-56, 1985.
- Winter and Perucho, "Oncogene Amplification during Tumorigenesis of Established Rat Fibroblasts Reversibly Transformed by Activated Human ras Oncogenes", *Molecular and Cellular Biology*, 6(7):2562-2570, 1986.
- Georges et al (1993) *Cancer Res* 53, 1743-1746.

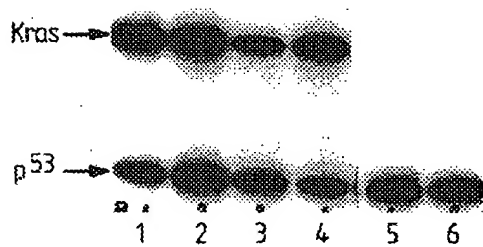
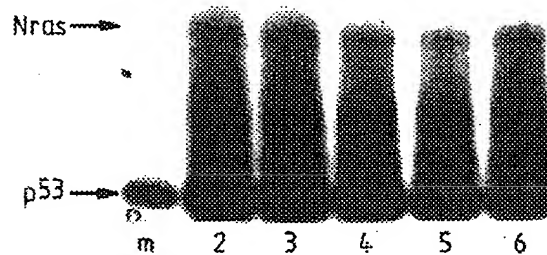
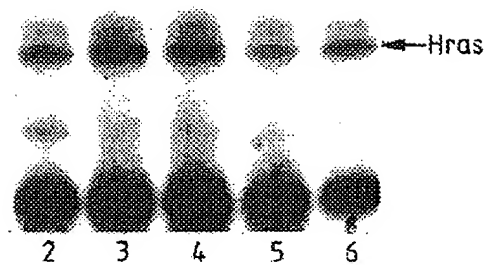
**FIG.1A-1****FIG.1A-2****FIG.1A-3****FIG.1A-4**

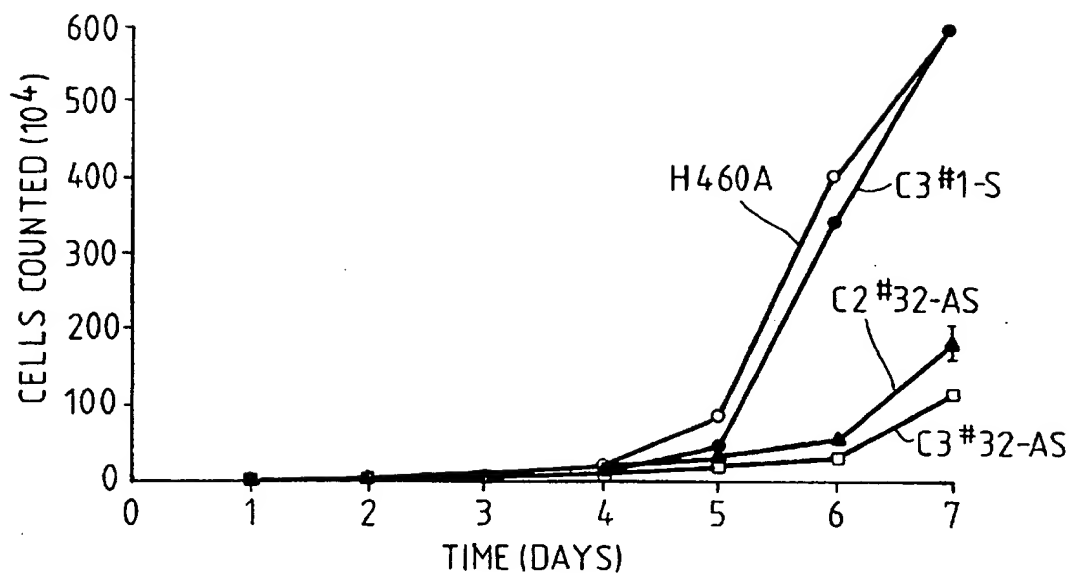
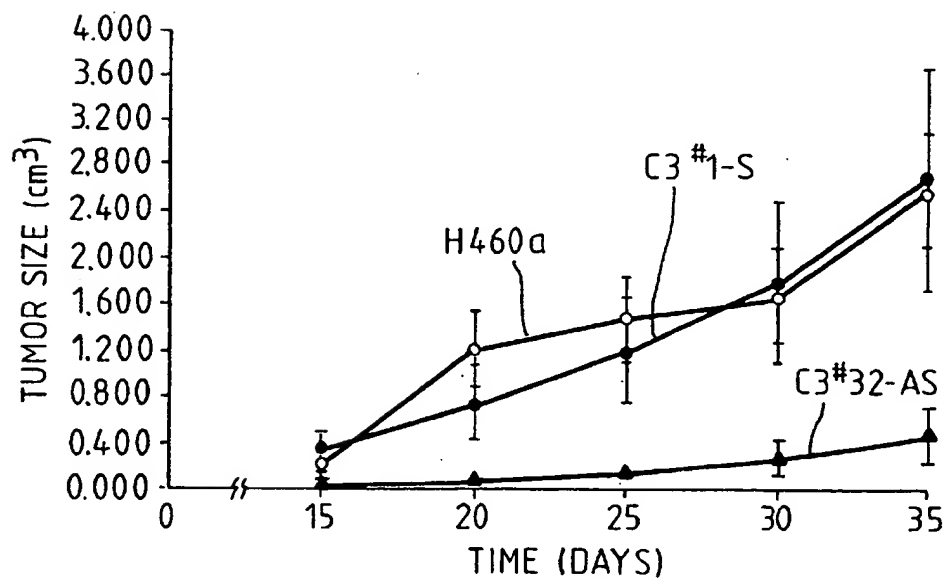
**FIG. 1B**

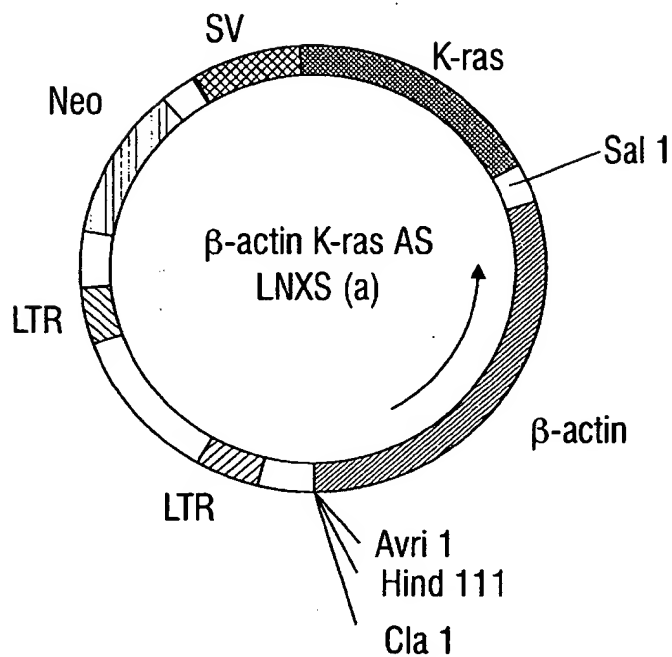
**FIG. 1C****FIG. 1D****FIG. 1E****FIG. 1F**

**FIG. 2A**

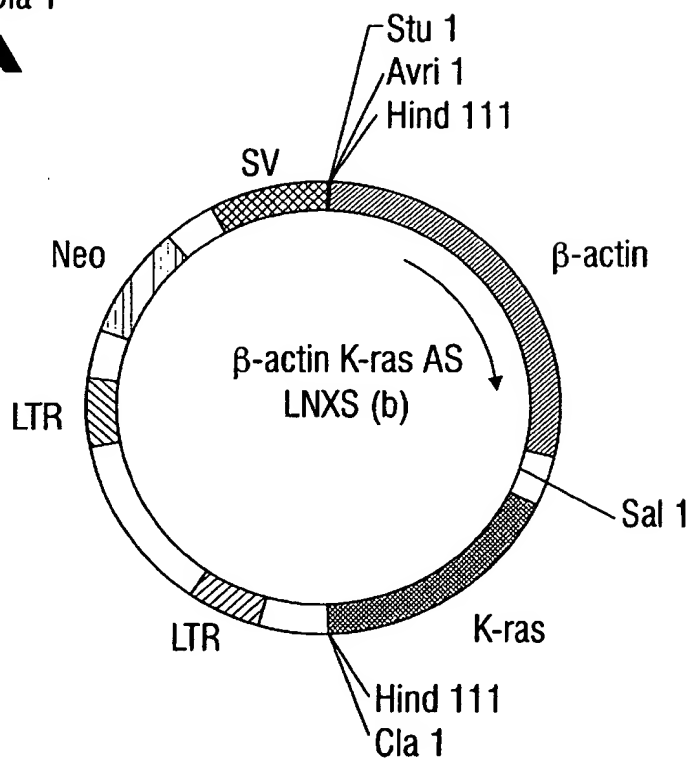


**FIG. 2B****FIG. 2C****FIG. 2D**

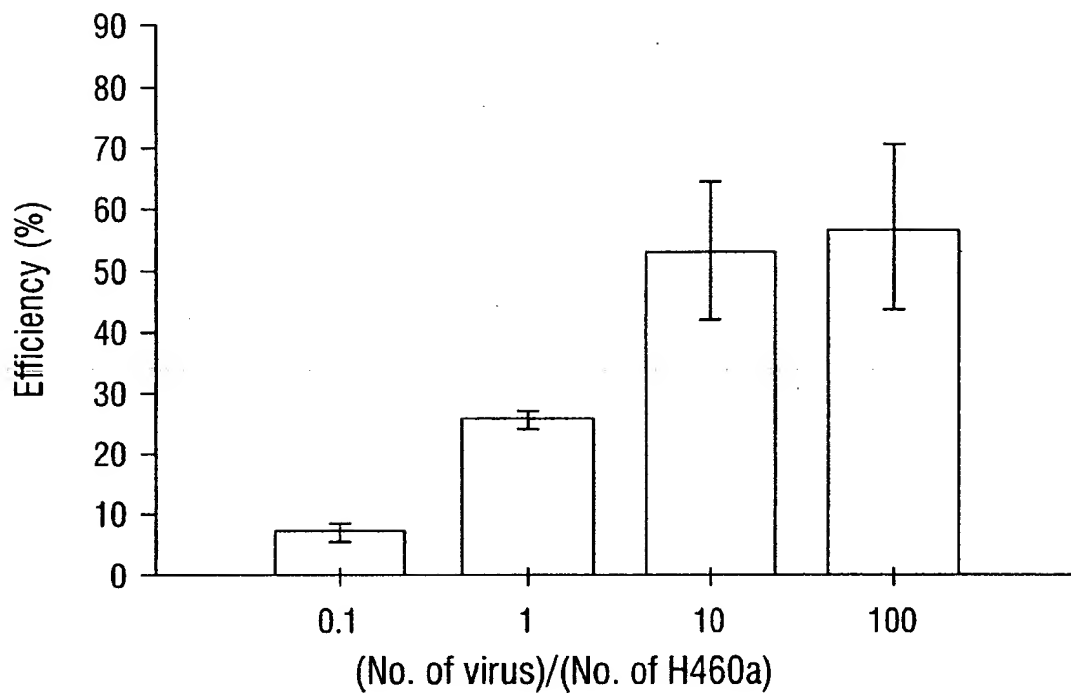
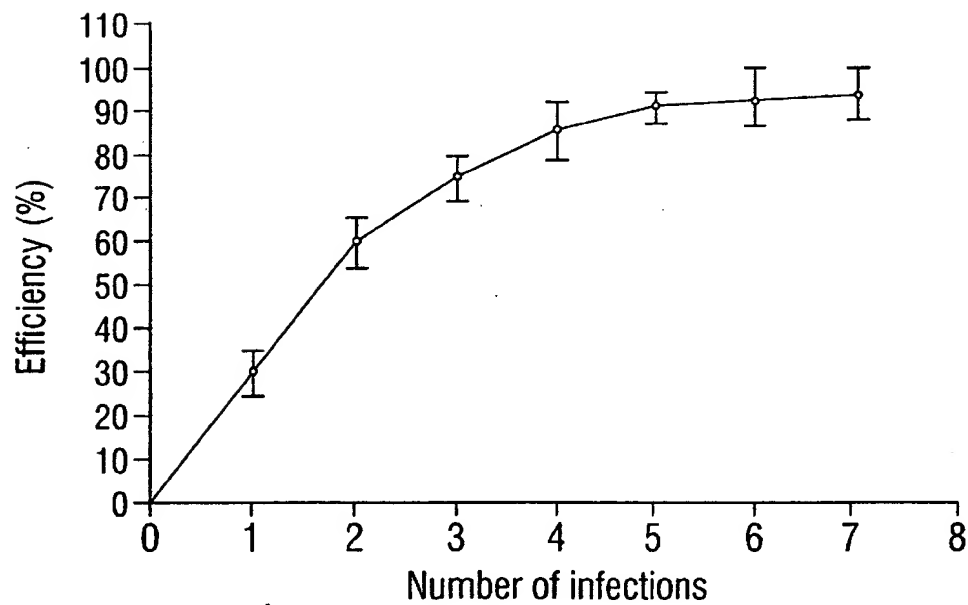
**FIG. 3A****FIG. 3B**

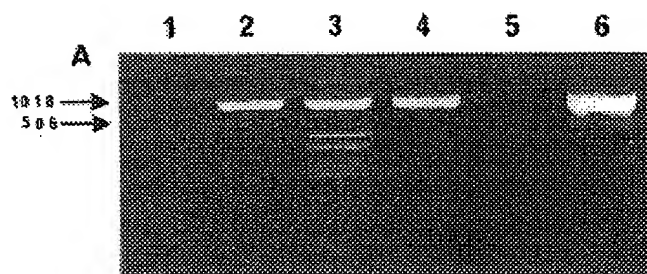
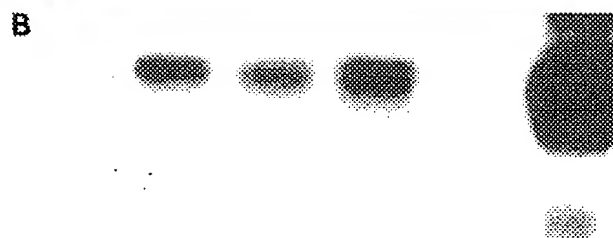


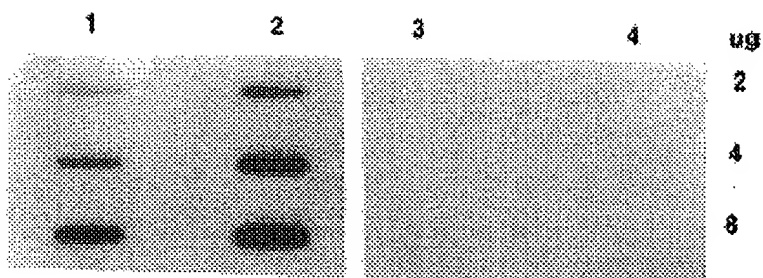
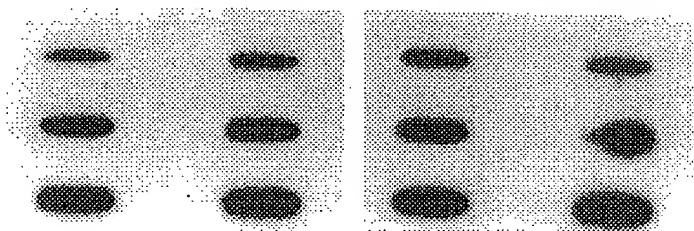
**FIG. 4A**

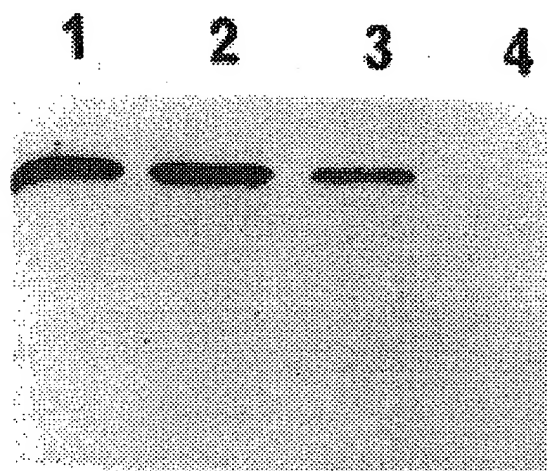
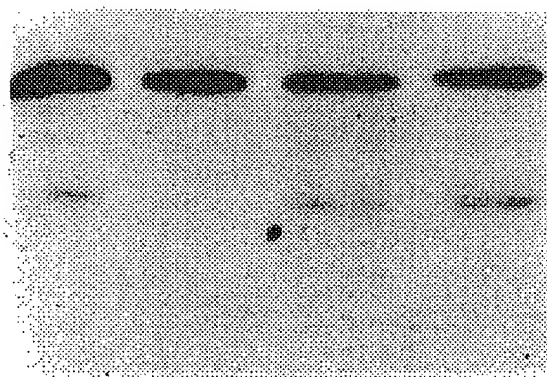


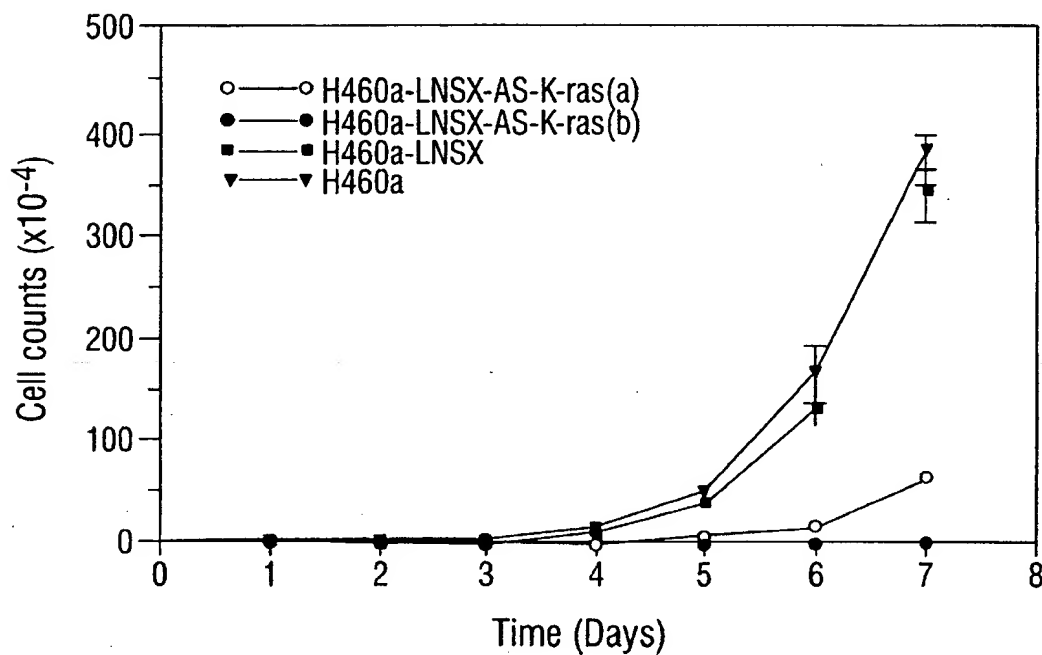
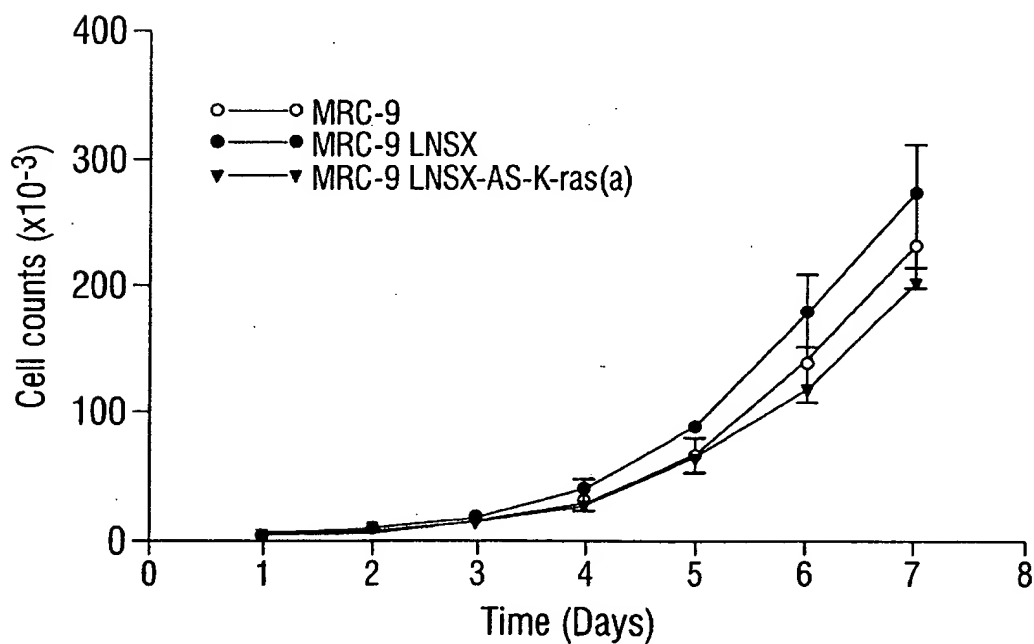
**FIG. 4B**

**FIG. 5A****FIG. 5B**

**FIG. 6A****FIG. 6B**

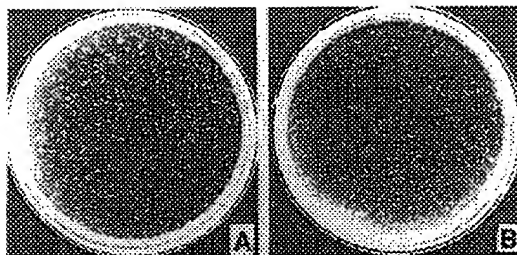
**FIG. 7A****FIG. 7B**

**FIG. 8A****FIG. 8B**

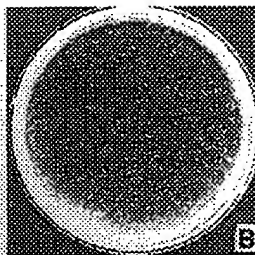
**FIG. 9A****FIG. 9B**



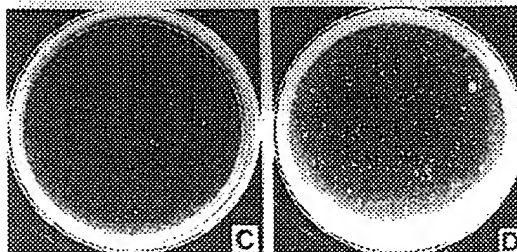
**FIG. 10A**



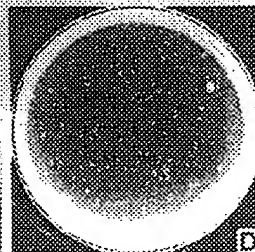
**FIG. 10B**

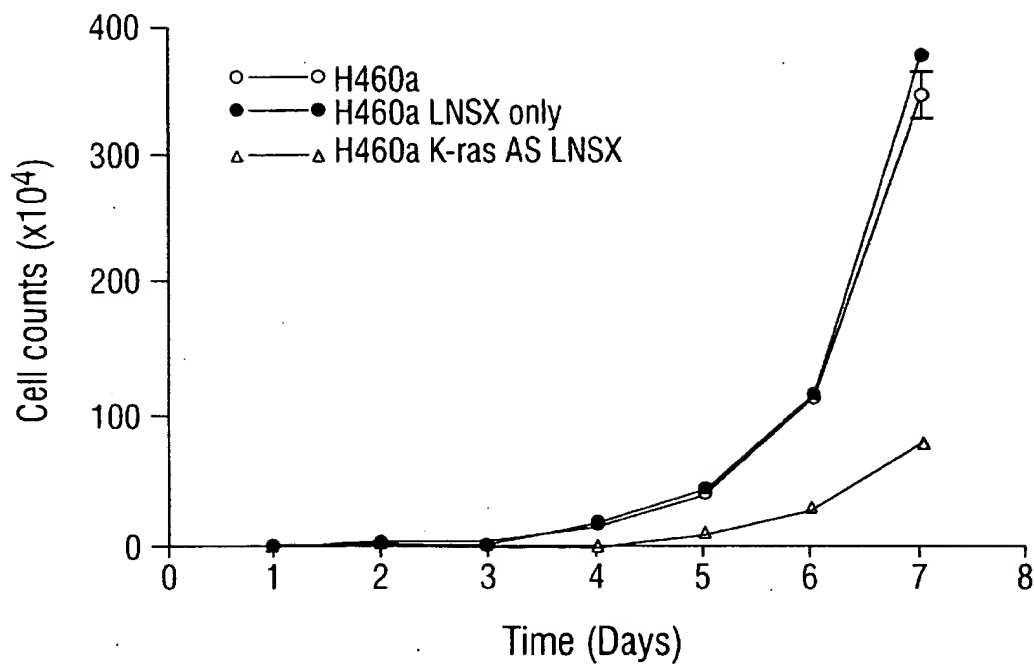
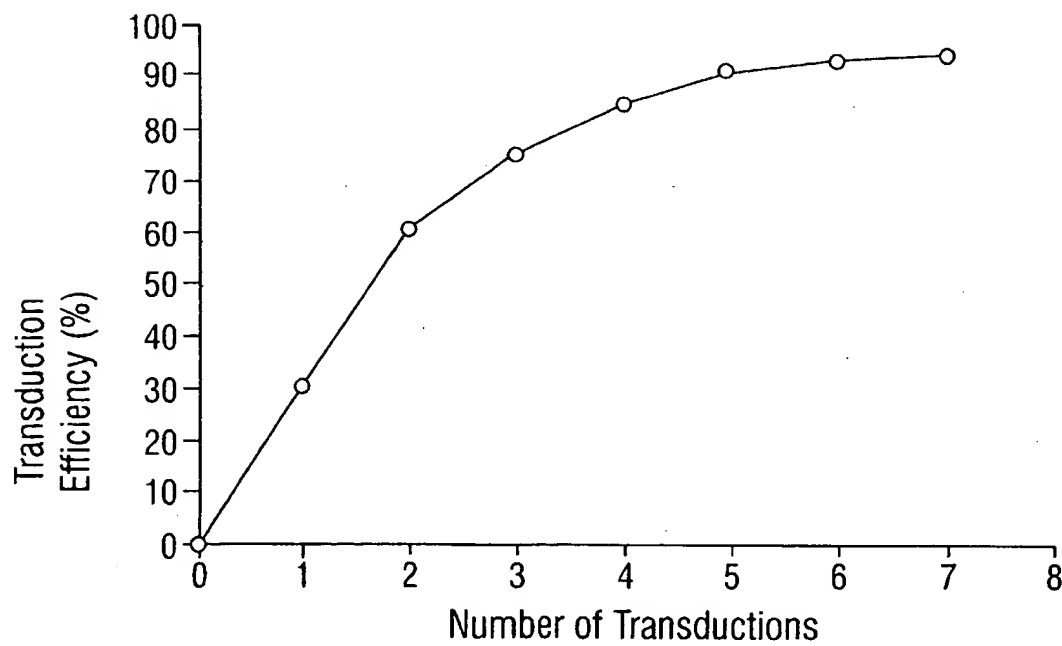


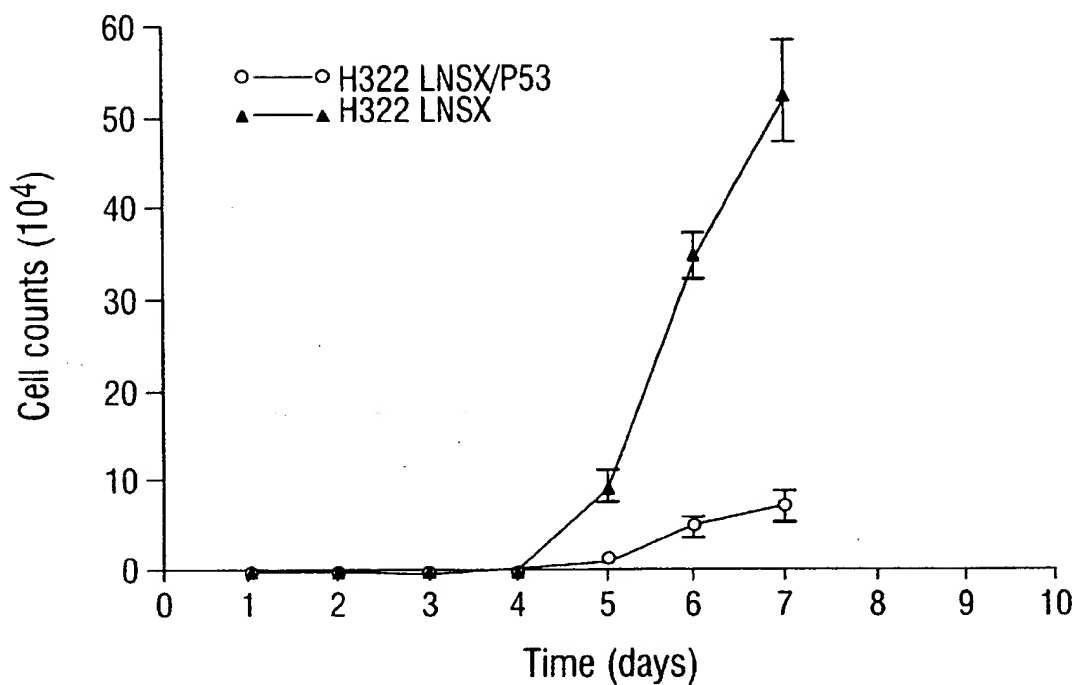
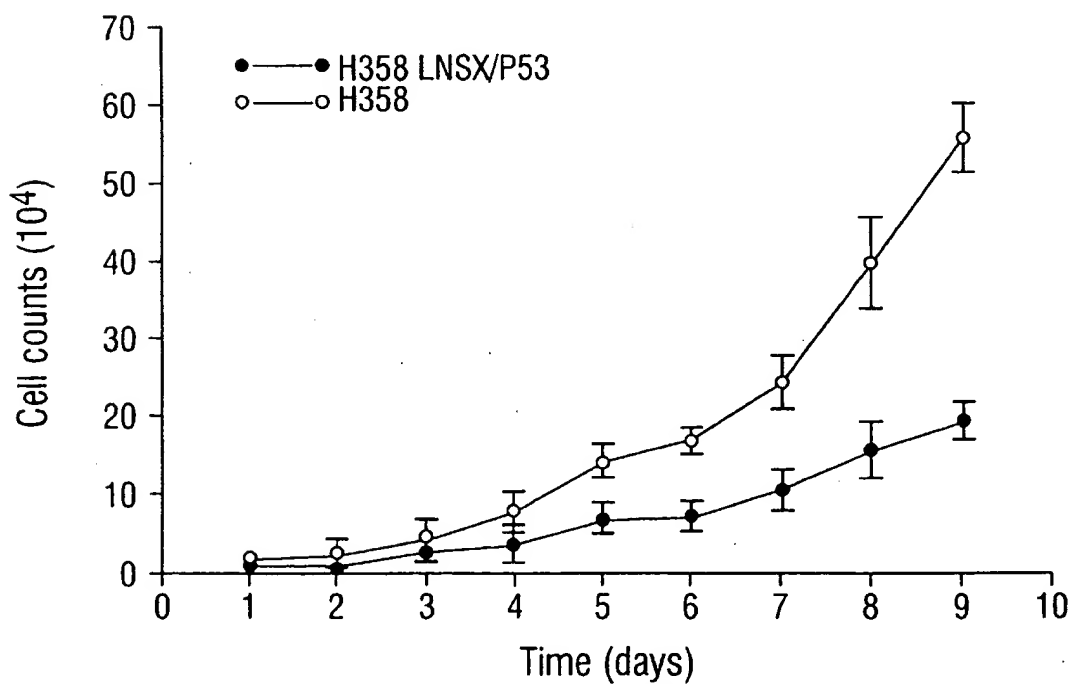
**FIG. 10C**



**FIG. 10D**



**FIG. 11****FIG. 12**

**FIG. 13****FIG. 14**

# INHIBITING THE GROWTH P53 DEFICIENT TUMOR CELLS BY ADMINISTERING THE P53 GENE

The present application is a continuation-in-part of U.S. Ser. No. 07/665,538, filed Mar. 6, 1991.

The government may own certain rights in the present invention pursuant to NIH grants RO1 CA 45187 and CA 16672.

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

The present invention relates to methods and nucleic acid vector compositions for modifying gene expressing, involving the preparation and use of improved retroviral vectors which encode antisense RNA molecules or, alternatively, transcriptionally active RNAs that encode selected proteins. The retroviral constructs of the present invention may be employed for introducing desired gene expression units into selected target cells, such as into tumor cells within individuals afflicted with cancer.

### 2. Description of the Related Art

It is now well established that a variety of diseases, ranging from certain cancers to various genetic defects, are caused, at least in part, by genetic abnormalities that result in either the over expression of one or more genes, or the expression of an abnormal or mutant gene or genes. For example, many forms of cancer in man are now known to be the result of, at least indirectly, the expression of "oncogenes". Oncogenes are genetically altered genes whose altered expression product somehow disrupts normal cellular function or control (Spandidos, et al., 1989).

Most oncogenes studied to date have been found to be "activated" as the result of a mutation, often a point mutation, in the coding region of a normal cellular gene or of a "protooncogene", that results in amino acid substitutions in the protein expression product. This altered expression product, in turn, exhibits an abnormal biological function that somehow takes part in the neoplastic process (Travali, et al., 1990). The underlying mutations can arise by various means, such as by chemical mutagenesis or ionizing radiation.

A number of oncogenes have now been identified and characterized to varying degrees, including ras, myc, neu, raf, erb, src, fms, jun and abl (Travali, et al., 1990; Minna, 1989; Bishop, 1987). It is likely that as our knowledge of tumorigenesis increases, additional oncogenes will be identified and characterized. Many of the foregoing, including ras, myc and erbB, comprise families of genes, whose expression product bear sequence similarities to other members of the family (Shih, et al., 1984; Bos, 1989; Schwab, et al., 1985). In the case of many of these gene families, it is typical that oncogenesis involves an activation of only one member of the family, with other "unactivated" members serving a role in normal cellular functions (Id.).

The study of DNA-mediated gene transfer has revealed the existence of activated cellular oncogenes in a variety of human tumors (for review, see Cooper, et al., 1982). Oncogenes have been identified in human bladder, colon, lung and mammary carcinoma cell lines (Krontiris, et al., 1981; Murray, et al., 1981; Perucho, et al., 1981), promyelocytic leukemia (Murray, et al., 1981), neuroblastoma (Shimizu, et al., 1983) and sarcoma cell lines (Pulciani, et al., 1982), and various solid tumors including carcinomas of the lung, and pancreas (Pulciani, et al., 1982). Studies have demonstrated that various transforming genes detected by transfection

correspond to activated cellular homologues of retroviral oncogenes (Pulciani, et al., 1982; Der, et al., 1982; Parada, et al., 1982; Santos, et al., 1982), although others have no known retroviral cognate (Tulciani, et al., 1982; Lane, et al., 1982).

The ras oncogene family has been perhaps the best characterized to date (Barbacid, 1987; Bos, 1989). Most of the identified transforming genes in human carcinomas have been a member of the ras gene family, which encode immunologically related proteins having a molecular weight of 21,000 (p21) (Ellis, et al., 1981; Papageorge, et al., 1982). This family is comprised of at least 3 members, one transduces as H-ras in the Harvey strain of murine sarcoma virus (Ellis, et al., 1981), one as K-ras and Kirsten murine sarcoma virus (Ellis, et al., 1981), and one identified by low stringency hybridization to H-ras, termed N-ras (Shimizu, et al., 1983). As noted, all members of the ras gene family encode closely related proteins of approximately 21,000 Daltons which have been designated p21s (Ellis, et al., 1981). The level of p21 expression is similar in many different human tumor cells, independent of whether the cell contains an activated ras gene detectable by transfection.

Nucleotide sequence analysis of the H-ras transforming gene of the EJ human bladder carcinoma has indicated that the transforming activity of this gene is a consequence of a point mutation altering amino acid 12 of p21 from glycine to valine (Tabin, et al., 1982). Studies of proteins encoded by K-ras genes activated in four human lung and colon carcinoma cell lines indicated that the transforming activity of K-ras in these human tumors was also a consequence of structural mutations (Der and Cooper, 1983). Other mutations have been found to result in ras gene activation as well. For example, the H-ras gene activated in a lung carcinoma cell line encodes the normal amino acid position 12 but is mutated at codon 61 to encode leucine rather than glutamine (Yuasa, et al., 1983). An N-ras gene activated in a human neuroblastoma cell line is also mutated at codon 61 but encodes lysine rather than glutamine (Taparowski, et al., 1983). Thus, studies such as these have indicated that ras genes in human neoplasms are commonly activated by structural mutations, often point mutations, that thus far occur at codon 12 or 61 with different amino acid substitutions resulting in ras gene activation in different tumors.

Antisense RNA technology has been developed as one approach to inhibiting gene expression, particularly oncogene expression. An "antisense" RNA molecule is one which contains the complement of, and can therefore hybridize with, protein-encoding RNAs of the cell. It is believed that the hybridization of antisense RNA to its cellular RNA complement can prevent expression of the cellular RNA, perhaps by limiting its translatability. While various studies have involved the processing of RNA or direct introduction of antisense RNA oligonucleotides to cells for the inhibition of gene expression (Brown, et al., 1989; Wickstrom, et al., 1988; Smith, et al., 1986; Buvoli, et al., 1987), the more common means of cellular introduction of antisense RNAs has been through the construction of recombinant vectors which will express antisense RNA once the vector is introduced into the cell.

A principal application of antisense RNA technology has been in connection with attempts to affect the expression of specific genes. For example, Delauney, et al. have reported the use antisense transcripts to inhibit gene expression in transgenic plants (Delauney, et al., 1988). These authors report the down-regulation of chloramphenicol acetyl transferase activity in tobacco plants transformed with CAT sequences through the application of antisense technology.

Antisense technology has also been applied in attempts to inhibit the expression of various oncogenes. For example, Kasid, et al., 1989, report the preparation of recombinant vector construct employing Craf-1 cDNA fragments in an antisense orientation, brought under the control of an adenovirus 2 late promoter. These authors report that the introduction of this recombinant construct into a human squamous carcinoma resulted in a greatly reduced tumorigenic potential relative to cells transfected with control sense transfectants. Similarly, Prochownik, et al., 1988, have reported the use of Cmyc antisense constructs to accelerate differentiation and inhibit G<sub>1</sub> progression in Friend Murine Erythroleukemia cells. In contrast, Khokha, et al., 1989, discloses the use of antisense RNAs to confer oncogenicity on 3T3 cells, through the use of antisense RNA to reduce murine tissue inhibitor or metalloproteinases levels.

Unfortunately, the use of current antisense technology often results in failure, particularly where one seeks to selectively inhibit a member of a gene family. One reason for this failure can be traced to the high expression levels of antisense message that are apparently required for inhibition. Unfortunately, the requisite expression levels of antisense message has not been generally achievable with existing constructs. Problems have also arisen due to the similarity in underlying DNA sequences, which results in the cross-hybridization of antisense RNA, retarding the expression of genes required for normal cellular functions. An example is presented by Debus, et al., 1990, who reported that in the case of ras oncogenes, antisense ras oligonucleotides kill both normal and cancer cells, which, of course, is not a desired effect.

Another important "oncogene" is the gene encoding the p53 cellular protein. The p53 gene is one of the most common targets for genetic abnormalities in human tumors (Hollstein et al., 1991). For example, it has been reported that p53 mutations occur in all histological types of lung cancer at frequencies of about 75% in small cell lung cancer (SCLC) and about 50% in non small cell lung cancer (NSCLC) (Takahashi et al., 1991). Evidence suggests that p53 acts as a "tumor suppressor" gene, and its inactivation through mutation can lead to oncogenic development. In fact, a predominance of G to T transversions in p53 and ras mutations in lung cancer, as well as epidemiological data, supports a close association between smoking and p53 mutations in NSCLC have suggested that p53 is a candidate for molecular targets of genetic damage caused by cigarette smoke (Zakut-Houri et al., 1985).

One approach that has been suggested as a means of treatment of such tumors is the introduction of so-called "wild-type" or non-mutated p53 (wt-p53) into affected cells, e.g., through the use of retroviral vectors which encode the wild type protein (Takahashi et al., 1992; Lee et al., EP appl. publ. 0 475 623 A1). The vectors proposed by these individuals included a wt-p53 genes wherein the direction of transcription of the encoded wt-p53 was in the same orientation as that of the retroviral long terminal repeats (LTRs). Unfortunately, in studies conducted by the present inventors reported hereinbelow, the ability of retroviral wt-p53 constructs prepared having such an orientation to suppress tumor growth was found to be less than optimal. Presumably, this shortcoming is the result of poor expression of the wt-p53 gene in the target cells.

Therefore, while it is clear that current gene therapy technology shows potential promise as a means of external control of gene expression, it is equally clear that it does suffer particular drawbacks, such as the need for high level expression and a lack of selectivity where gene families are

concerned. There is a particular need, therefore, for a general approach to the design of gene therapy protocols that will allow selective inhibition of gene expression, even in the case of closely related genes.

#### SUMMARY OF THE INVENTION

The present invention, in a general and overall sense, addresses one or more of the foregoing or other shortcomings in the prior art by providing a novel approach to the design of retroviral vectors for the intracellular delivery of selected genetic constructs in a manner which allows their use to inhibit the expression of specific genes, or to replace defective genes, in target cells.

The inventors believe that the approach offered by the present invention offers more specificity and selectivity than previous approaches. Additionally, it is proposed that the present invention will allow that the development of vector technology for gene therapy having a much improved ability to inhibit or provide for specific gene expression, particularly in those instances where one desires to selectively inhibit a particular gene over that of closely related genes or other members of a gene family, or where one desires to provide for the expression of a specific gene.

A particularly surprising aspect of the invention, discussed in more detail below, is the finding that by aligning the selected promoter/gene construct within the vector in an orientation that is reversed with respect to the direction of transcription of other promoters within the vector, one can achieve a dramatic increase in transcription of the introduced gene. Thus, where retroviral vectors are employed, the promoter/gene construct should be aligned so as to effect transcription in a direction that is opposite that of usual viral transcription. In the case of retroviruses, a reverse orientation is one that is opposite that of long terminal repeat transcription. While this affect was observed using the  $\beta$ -actin promoter and a retroviral expression vector, the inventors believe that this phenomenon will be applicable to other promoter/vector constructs for application in gene therapy.

In one specific embodiment, the invention concerns vector constructs for introducing wild type p53 genes (wt-p53) into affected target cells suspected of having mutant p53 genes. These embodiments involve the preparation of a gene expression unit wherein the wt-p53 gene is placed under the control of the  $\beta$ -actin promoter, and the unit is positioned in a reverse orientation into a retroviral vector.

While aspects of the invention are exemplified through the use of wt-p53 constructs, and their use in cancer treatment, it is proposed that the invention is generally applicable to any situation where one desires to achieve high level expression of a recombinant protein in a target or host cell through the use of a retroviral expression vector. This could, for example, involve the use of a gene encoding a recombinant protein that confers a particular trait, such as the use of wt-p53 to "replace" a trait that has been lost due to mutation, or could be used to introduce protein-encoding genes that one desires to use for preparing proteins for other purposes, such as in recombinant protein production procedures. While the nature of the gene introduced is not critical to broader aspects of the invention, it should be mentioned that in the context of cancer treatment modalities, a particular example in addition to p53 replacement that is contemplated by the inventors is the introduction of the retinoblastoma gene (rb).

In embodiments where inhibition or suppression of gene expression is desired, antisense molecules will be employed.

By preparing a construct that encodes an RNA molecule that is in antisense or "complementary" configuration with respect to the RNA readouts of the target gene, the construct will act to inhibit or suppress the ultimate expression of the target gene, presumably by binding to the target RNA and thereby preventing its translation. In that the novel aspects of this part of the invention concerns the discovery of an improved retroviral promoter construct, the invention is generally applicable to any antisense construct.

For certain applications in the context of antisense constructs, therefore, the antisense RNA that is produced will be complementary to a selected cellular gene, such as an oncogene sequence or some other sequence whose expression one seeks to diminish through antisense application. While all or part of the coding sequence may be employed in the context of antisense construction, the inventors have found that particular advantage may be taken where one employs in the antisense construct an intron-complementary region that will bind to transcribed introns contained in the targeted RNA. It has been found that the use of intron-complementary regions not only improves the inherent inhibitory characteristics of the antisense molecule, but it also provides one the ability to selectively inhibit one member of a gene family over another. This is due to the fact that while exon regions of members of gene families will often be similar, it is typically the case that the intron regions of these genes will be different.

Thus, in preferred aspects of the invention, antisense molecules will include a region that is complementary to and is capable of hybridizing with an intron region of the gene whose expression is to be inhibited. The inclusion of intron-complementary regions in the antisense RNA constructs of the present invention is believed to be the key to both an improved inhibitory capability as well as selectivity. By way of theory, it is proposed that the use of antisense intron regions provides an improved capability for at least two reasons. It is known that the structure of intron RNA plays a role in RNA processing.

The inventors propose that antisense introns bind to "sense" intron regions found on the initial RNA transcript of the gene, and affects proper RNA processing. Thus, subsequent translation of protein-coding RNAs into their corresponding proteins is retarded or prevented. The use of antisense introns are believed to provide selectivity of inhibition because the exon or "amino acid encoding" region of RNAs coding for closely related proteins are often themselves closely related. This may not be the case for the introns of closely related genes. Thus, where intron regions between two genes are distinct, antisense introns can be designed which will hybridize selectively to a selected gene family member, and not to other family members, and thereby inhibit selectivity.

As used herein, the term "intron" is intended to refer to gene regions that are transcribed into RNA molecules, but processed out of the RNA before the RNA is translated into a protein. In contrast, "exon" regions of genes are those regions which are transcribed into RNA and subsequently translated into proteins.

Thus, where one seeks to selectively inhibit a particular gene or genes over a related gene or genes, the inventors propose the preparation and use of antisense RNA molecules which encode an intron region or regions of the gene which one desires to inhibit selectively, that is distinct from intron regions of genes which one desires to leave unaffected. A "distinct" intron region, as used herein, is intended to refer to an intron region that is sufficiently different from an intron

region of another gene such that no cross hybridization would occur under physiologic conditions. Typically, where one intron exhibits a sequence homology of no more than 20% with respect to a second intron, one would not expect hybridization to occur between antisense and sense introns under physiologic conditions.

While it is generally preferred that antisense introns be prepared to be complementary to an entire intron of the gene to be inhibited, it is believed that shorter regions of complementarity can be employed, so long as the antisense construct can be shown in vitro to inhibit expression of the targeted expression product. The inventors believe that the most important intron regions in terms of the preparation of antisense introns will be those regions closest to intron/exon junctions. This is the region where RNA processing takes place. Thus, it is proposed that one will desire to include it in the antisense intron sufficient complementarity with regions within 50-100 nucleotides of the intron/exon junction.

The inventors have found that some antisense exon sequences of the targeted gene can also be included in the antisense constructs of the present invention, so long as the resultant construct maintains its selectivity, and will not seriously inhibit genes whose continued function is relied upon by the cell for normal cellular activities. The amount of antisense exon sequence included within the antisense construct which can be tolerated will likely vary, depending on the particular application envisioned. For example, antisense constructs for down-regulation of K-ras expression have been prepared which include sequences complementary to exons II and III and all of intron II of the K-ras gene. These constructs contain antisense sequences to intron II of K-ras, and selectively inhibit K-ras expression relative to H-ras or N-ras. Thus, in this instance, the inclusion of sequences complementary to exons II and III of K-ras apparently did not result in the significant inhibition of the H-ras or N-ras genes, even though a 300 nucleotide region of complementarity existed with exons of the unaffected genes.

One can readily test whether too much antisense exon DNA has been included in antisense intron constructs of the present invention by simply testing the constructs in vitro to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences are affected.

In connection with these aspects of the invention, it is proposed that the antisense constructs of the present invention, whether they be the antisense RNA molecules (i.e., oligonucleotides) or nucleic acid molecules which encode for antisense RNA molecules, will have their principal application in connection with the down-regulation of oncogene expression.

The most preferred oncogenes for application of the present invention will be those which exist as a family of genes, where one desires to selectively inhibit one member of a family over other members. In this regard, one may mention by way of example, the ras, myc, erb or jun families of oncogenes. Certain of these, such as the ras family, involves the activation of protooncogenes by a point mutation, which apparently results in the expression of a biologically abnormal product.

In aspects that relate to the use of intron sequences, the present invention contemplates that antisense intron RNA can either be applied directly to cells, in the form of oligonucleotides incorporating antisense intron sequences, or by introducing into the cell nucleic acid sequences that

will encode the desired antisense construct in the form of retroviral constructs. In the former case, it has been shown by others that antisense oligonucleotides can successfully traverse cellular membranes. The present inventors envision that such an approach may be an option to therapy, particularly where the antisense oligonucleotides are successfully packaged to maintain their stability in circulation, for example, by liposome encapsulation.

Other techniques for direct insertion in the cells include, by way of example, electroporation, or calcium phosphate transfection. Furthermore, where one desires to treat conditions of the bone marrow, bone marrow cells can be successfully removed from the body, treated with antisense constructs, and replaced into the body similar to the adoptive immunotherapy approach employed in IL-2 treatment.

In broader aspects of the invention, a preferred approach will involve the preparation of retroviral vectors which incorporate nucleic acid sequences encoding the desired construct, once introduced into the cells to be treated, preferably, these sequences are stably integrated into the genome of the cell. One example of such of vector construct would be a replication defective retrovirus, such as LNSX, LN or N2A, that is made infective by appropriate packaging, such as by GPlenvAM12 cells. Although the retrovirus would inhibit the growth of the tumor, the expression of the antisense construct in non-tumor cells would be essentially harmless where one prepares a retrovirus construct which encode distinct antisense intron RNA in accordance with the present invention. In addition to retroviruses, it is contemplated that other vectors can be employed, including adenovirus, adeno-associated virus, or vaccinia viruses (Hermonat, et al., 1984; Karlsson, et al., 1985; Mason, et al., 1990).

The particular promoter that is employed to control the expression of the antisense RNA in a vector construct is not believed to be particularly crucial, so long as it is capable of expressing the antisense intron RNA in the targeted cell of a rate greater than 5 fold that of the gene to be inhibited. Thus, where a human cell is targeted, it will be preferred to position the antisense RNA coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human cellular or viral promoter. While the  $\beta$ -actin promoter is preferred the invention is by no means limited to this promoter, and one may also mention by way of example promoters derived from RSV, N2A, LN, LNSX, LNSN, SV40, LNCX or CMV (Miller, et al., 1989; Hamtzoopoulos, et al., 1989).

The most preferred promoters will be those that are capable of being expressed in a wide variety of histologic cell types, and which is capable of continuously expressing the antisense RNA. A preferred example is the  $\beta$ -actin promoter, because the promoter functions effectively in human epithelial cells. Other examples of promoters having a similar capability include RSV and SV40.

Where retroviral vectors are concerned, a more particular feature of the present invention is the general, overall design of preferred retroviral vector constructs. The most preferred vector design of the present invention takes into account the inventors' discovery that when a particular promoter, the  $\beta$ -actin promoter, is employed to drive expression of a selected gene, and the expression construct is positioned in an orientation that is opposite that of retroviral transcription, there is a surprising increase in the relative expression of the selected gene. Thus, generally speaking, retroviral constructs of the present invention can be said to include a gene

expression unit which includes a selected gene under the control of a  $\beta$ -actin promoter, wherein the gene expression unit is positioned to effect transcription of the selected gene in an orientation opposite that of retroviral transcription.

By "reverse orientation" or "opposite orientation" is meant that the orientation of transcription of the selected gene that is under the control of the  $\beta$ -actin promoter is in the opposite direction from the direction of transcription of the regular retroviral genes. Thus, for example, where the vector includes a long terminal repeat (LTR), as do most retroviral vectors, the orientation of transcription of the selected gene will be opposite that of the LTR.

While the retroviral construct aspect of the present invention concerns the use of a  $\beta$ -actin promoter in reverse orientation, there is no limitation on the nature of the selected gene which one desires to have expressed. Thus, the invention concerns the use of antisense-encoding constructs as well as "sense" constructs that encode a desired protein.

Of particular importance is the inventors somewhat surprising discovery that reversing the orientation of the genetic construct with respect to the direction of transcription of the retroviral vector dramatically improves expression of the selected gene. This effect is dramatically illustrated in the context of K-ras antisense therapy (see FIG. 9A and Example II below). In these studies, when the antisense construct was expressed from a retroviral vector aligned in the same direction of transcription as the retroviral LTR, the effect in suppressing target cells versus control cell growth was evident, but target cells growth was nonetheless observed by 7 days. In stark contrast, no growth was observed after 7 days where the reverse orientation construct was employed.

The nature of the retroviral vector that is employed may depend upon the application that is envisioned. For clinical application, there are several types of such vectors that have been found or proposed as applicable, such as a Moloney murine leukemia virus vector, mouse mammary tumor virus, or related retroviruses, or the like. The use of these vectors for clinical application rests upon the fact that they do not include active viral genes that could be considered harmful to humans or animals and do not lead to the production of infective viruses upon infection. However, the invention is not limited in its scope to clinical applications, and for applications that do not contemplate clinical administration to humans or animals it is proposed that virtually any type of retrovirus can be employed.

Certain preferred vectors designed and employed by the present inventors will include a second gene expression unit which includes a second gene, such as a selectable marker gene, expressed from a retroviral long-term repeat. The presence of a selectable marker genes facilitate the preparation of the vector by allowing the selection of appropriate host cells from which the vector is prepared. The nature of the marker gene is not believed to be particularly crucial, so long as it does not produce a product that is harmful to the host cell, or to humans or animals where clinical application is contemplated.

Where clinical application of retroviral vectors is contemplated, it will be necessary to prepare the vector and place it into a pharmaceutical composition that is appropriate for the intended application. This will entail generally preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One will also generally desire to employ appropriate salts and buffers to render the vector stable and allow for vector uptake by target cells. The

preparation of appropriate pharmaceutical retroviral compositions are generally well known, as are appropriate amounts, etc., of vectors to be employed.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A-1, FIG. 1A-2, FIG. 1A-3, FIG. 1A-4, FIG. 1B, FIG. 1C, FIG. 1D, FIG. 1E, FIG. 1F and FIG. 1G. FIG. 1A-1, FIG. 1A-2, FIG. 1A-3 and FIG. 1A-4. The second exon of the K-ras gene was amplified from genomic DNA of H522, H322, Calu 1, H226, H460a and human placenta by polymerase chain reaction (PCR), blotted onto a gene screen membrane and hybridized with  $^{32}$ P end-labeled oligonucleotide probes. FIG. 1A-1 shows the presence of wild-type glutamine residue (CAA) at 61 codon in five cell lines except H460a. The same blot was reprobed with a histidine-specific mutated oligo probe (CAT) and only the H460a cell line PCR DNA hybridized (FIG. 1A-2). The mutation was confirmed by direct PCR DNA sequencing. Wild-type K-ras 61 codon sequence in human placenta (FIG. 1A-3) was compared with the H460a cell line (FIG. 1A-4).

FIG. 1B. A 2 kb genomic DNA segment from the K-ras oncogene was subcloned into in Apr-1-neo vector in both a sense and antisense orientation. A 2 kb Eco RI/Pst I fragment containing second and third exon sequences together with adjoining flanking intron sequences was isolated from the SP6 vector (Oncogene Sciences) and Klenow enzyme was used to make blunt ends. Apr-1-neo vector was digested with Bam HI and blunt end ligation was performed to obtain the Apr-1-neo AS or Apr-1-neo A constructs.

FIG. 1C. A southern blot analysis of the K-ras oncogene in H460a and H460a transfectants. Blots were probed with P32 nick translated 2 kb Eco RI/Pst I insert DNA. 1) H460a, (2,3) H460a transfected with Apr-1-neo S C<sub>1</sub>#1 and C<sub>2</sub>#1 (4,5) H460a cells transfected with Apr-1-neo AS, C<sub>3</sub>#32 and C<sub>2</sub>#32, respectively.

FIG. 1D. A northern blot analysis of sense and antisense K-ras RNA. 1) H460a, (2,3) Apr-1-neo S transfectants, (4,5) Apr-1-neo AS transfected clones.

FIGS. 1E and 1F. A Western blot analysis of K-ras specific p21-protein FIG. 1E and total ras protein FIG. 1F was performed using either pan ras or K-ras specific monoclonal antibodies. 1) Calu-1 control cell line over expressing K-ras specific protein. 2) H460a; 3) H460a Apr-1-neo S; 4,5) H460a Apr-1-neo AS.

FIG. 1G Map of plasmid pH  $\beta$  Apr-1-neo

FIG. 2A, FIG. 2B, FIG. 2C and FIG. 2D. FIG. 2A. Schematic diagram of K-ras RNA synthesis. A segment of ras cDNA was amplified using oligonucleotide primers corresponding to the 5' region of first exon and 3' of second exon (indicated by arrows) for RNA PCR analysis.

FIG. 2B. An RNA PCR analysis was done to compare the level of K-ras message in H460a and H460a transfectants. As a control, a portion of p53 gene was co-amplified with p53 specific primer which served as an internal control.

FIG. 2C and FIG. 2D. H-ras and N-ras specific amplimers were used to quantitate H-ras/N-ras RNA in the transfectants and parental cell lines. p53 gene amplification is shown as an internal control.

FIG. 3A and FIG. 3B. FIG. 3A. In vitro growth curve. Cells were seeded at  $10^4$  cells/plate and grown for a seven day period. Cells were harvested and counted in a hemocytometer at 24 h intervals. Growth curves for H460A and H460A cells transfected with Apr-1-neo S vector do not show any significant difference, but H460A transfectants carrying Apr-1-neo-AS showed growth inhibition (FIG. 3B).

Female BALB/C nu/nu mice were injected with  $10^6$  H460a cells subcutaneously in the left flank. Cross-sectional diameters of the external tumor were measured without knowledge of the cell group. Tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Palpable tumors were first detected on day 15. Each point represents the mean  $\pm$ SE. C3#32-AS (n=5), C3#1-S (n=5), H460a (n=3). C3#32-AS was compared to C3#1-S or H460a on days 20, 25, 30, 35 ( $p < 0.05$  by Wilcoxon's Test).

FIG. 4A and FIG. 4B. Subcloning of  $\beta$ -actin K-ras antisense fragment in the LNSX retroviral vector. A 1.8-Kb genomic K-ras DNA segment with a 4-Kb  $\beta$ -actin promoter in antisense orientation was subcloned into a 6-Kb LNSX retroviral vector using blunt (a) or Hind III linker (b) ligations in two orientations.

FIG. 5A and FIG. 5B. LNSX-antisense (a) retrovirus infection efficiency in H460a cells. FIG. 5A. H460a cells  $10^5$  in 6-well plates were infected once with 1 ml of each serial dilutions of retroviral stocks in the presence of 8  $\mu$ g/ml polybrene. Two days later, seeding equal numbers of H460a transduced cells into 300  $\mu$ g/ml G418 selective medium or nonselective medium for 10-14 d. Infection efficiency=(No. of colonies in G418 medium)/(No. of colonies in medium without G418). FIG. 5B. H460a cells  $10^4$  in 12-well plates were incubated with 0.5 ml LNSX-antisense (orientation a) retroviral stocks (Titer:  $2 \times 10^6$  CFU/ml). Polybrene 8  $\mu$ g/ml was also added. The infections were done once each day for 1 to 7 d. Fresh medium and supernatant were added at each time point. The infection efficiency was calculated as for A.

FIG. 6A and FIG. 6B. PCR analysis of transduced H460a cells. The genomic DNA of H460a was extracted and amplified by PCR with neo 1 and neo 5 oligonucleotide primers. The PCR products were electrophoresed on 2% ethidium bromide-stained agarose gel (FIG. 6A). The DNA was transferred onto nitrocellulose membranes and hybridized with  $^{32}$ P-nick-translated neo gene probe (FIG. 6B). Lane 1: molecular weight marker; Lane 2: H460a-antisense LNSX (orientation a); Lane 3: H460a-antisense-LNSX (orientation b); Lane 4: H460a-LNSX; Lane 5: parental H460a; Lane 6: LNSX vector plasmid DNA.

FIG. 7A and FIG. 7B. Slot blot hybridization of poly(A+) RNA of H460a cells. Poly (A+) RNA was extracted, spotted onto nitrocellulose membranes (8  $\mu$ g, 4  $\mu$ g, or 2  $\mu$ g) and hybridized with  $^{32}$ P-end-labeled 42 bp K-ras exon 2 sense oligonucleotide probe (FIG. 7A). The filter was reprobed with a  $^{32}$ P-nick-translated  $\beta$ -actin probe to check for equal loading (FIG. 7B). Lane 1: H460a-antisense-LNSX (orientation b); Lane 2: H460a-antisense-LNSX (orientation a); Lane 3: H460a-LNSX; Lane 4: H460a parental cells.

FIG. 8A and FIG. 8B. Western blot analysis of ras p21 proteins in H460a cells. One hundred micrograms of protein was size fractionated by 12.5% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. K-ras-p21-specific (FIG. 8A) and pan-ras-specific monoclonal antibodies (FIG. 8B) were used, followed by HRP-labeled goat anti-mouse second antibody. Lane 1: H460a parental cells; Lane 2: H460a-LNSX; Lane 3: H460a-antisense-LNSX (orientation b); Lane 4: H460a-antisense-LNSX (orientation a).

FIG. 9A and FIG. 9B. FIG. 9A. Growth curve of H460a cells in vitro. Cells  $10^3$ /well were seeded in 12-well plates and grown for 7 days. Cells were harvested and counted daily by trypan blue exclusion. FIG. 9B. Growth curve of MRC-9 cells in vitro.



FIG. 10A, FIG. 10B, FIG. 10C and FIG. 10D. Soft agarose colony formation of H460a cells. Cells  $5 \times 10^4$  were mixed with 0.35% agarose in RPMI 1640 route medium and plated over a base layer of 0.7% agarose and culture medium hardened in 60-mm dishes. Colonies were counted 10–14 d later. FIG. 10A. Parental H460a; FIG. 10B. H460a-LNSX; FIG. 10C. H460a-antisense-LNSX (orientation a); FIG. 10D. H460a-antisense-LNSX (orientation b).

FIG. 11 Functional transduction efficiency of LNSX-AS-K-ras in H460a cells. Growth curves are shown for  $10^3$  cells/well seeded in 12 well plates. H460a cells were infected by incubation 0.5 m of viral supernatant stock from either LNSX or LNSX-AS-K-ras ( $6 \times 10^6$  CFU/ml) daily for 4 consecutive days in the presence of 8  $\mu$ g/ml of polybrene. The parental H460a cells served as a control. Cells were not selected with G418. Cells were counted daily. The mean  $\pm$ SE is shown for 3 replicates.

FIG. 12 H460a cells were infected with LNSX-AS-K-ras by incubating  $10^4$  cells with 0.5 ml of viral stock ( $6 \times 10^6$  CFU/ml) produced by the packaging cell line GP+envAm12 in the presence of 8  $\mu$ g/ml of polybrene. Infection was done daily for 1 to 7 days. Two days later cells were plated in equal numbers into selective media containing 200  $\mu$ g/ml G418. Control H460a cells were plated at equal cell numbers to determine baseline colony forming efficiency. The infection efficiency was measured by determining the percent of the unselected colony number formed by the G418 selected colonies.

FIG. 13 Growth curves are shown for  $10^4$  cells/well seeded in 12 well plates. H322a cells were infected by incubation 0.5 m of viral supernatant stock from either LNSX, DC, LNSX-p53 or DC-p53 ( $10^6$  CFU/ml) on 2 consecutive days in the presence of 8  $\mu$ g/ml of polybrene. The parental H322a cells served as a control. Cells were not selected with G418. Cells were counted daily. The mean  $\pm$ SE is shown for three replicates.

FIG. 14 Growth curves are shown for  $10^4$  cells/well seeded in 12 well plates. H460a cells were infected by incubation 0.5 m of viral supernatant stock from either LNSX, DC, LNSX-p53 or DC-p53 ( $10^6$  CFU/ml) in the presence of 8  $\mu$ g/ml of polybrene. The parental H322a cells served as a control. Cells were not selected with G418. Cells were counted daily. The mean  $\pm$ SE is shown for three replicates.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### Molecular Events in Lung Cancer Development

Lung cancer remains the leading cause of cancer deaths in the United States where it kills more than 140,000 people annually. Recently, age-adjusted mortality from lung cancer has surpassed that from breast cancer in women. Although implementation of smoking-reduction programs has decreased the prevalence of smoking, lung cancer mortality rates will remain high well into the 21st century (Brown et al., 1988). Unfortunately, all current treatment modalities, including radiation therapy, surgery, and chemotherapy, have limited effectiveness. The rational development of new therapies for lung cancer will depend on an understanding of the biology of lung cancer at the molecular level. Research carried out in the laboratories of the present inventors has identified critical molecular events leading to NSCLC development and progression. The goal of this research is to directly modify the cancer cell to eliminate the expression of gene products which are responsible for the maintenance or progression of the malignant phenotype or to restore in

normal form deleted or mutated gene products that suppress the characteristics of the malignant phenotype.

The most common lung cancer histologies (80%) are grouped under the term non-small-cell lung cancer (NSCLC) and include squamous, adenocarcinoma, and large-cell undifferentiated. Many of the current data on the molecular biology of lung cancer come from the study of the more uncommon small-cell lung cancer (SCLC). SCLC can be distinguished from NSCLC by the neuroendocrine features of the cells; SCLC is very responsive to chemotherapy but recurs rapidly after treatment. NSCLC also may serve as a model for other carcinogen-induced epithelial cancers. The approaches and observations developed in this study may be applicable to other types of epithelial cancers.

Abundant evidence has accumulated that the process of malignant transformation is mediated by a genetic paradigm (Bishop et al., 1991). The major lesions detected in cancer cells occur in dominant oncogenes and tumor suppressor genes. Dominant oncogenes have alterations in a class of genes called proto-oncogenes, which participate in critical normal cell functions, including signal transduction and transcription. Primary modifications in the dominant oncogenes that confer the ability to transform include point mutations, translocations, rearrangements, and amplification. Tumor suppressor genes appear to require homozygous loss of function, by mutation, deletion, or a combination of these for transformation to occur. Some tumor suppressor genes appear to play a role in the governance of proliferation by regulation of transcription. It is possible that modification of the expression of dominant and tumor suppressor oncogenes may influence certain characteristics of cells that contribute to the malignant phenotype.

Despite increasing knowledge of the mechanisms involved in oncogene-mediated transformation, little progress has occurred in developing therapeutic strategies that specifically target oncogenes and their products. Initially, research in this area was focused on dominant oncogenes; as these were the first to be characterized. DNA-mediated gene transfer studies showed acquisition of the malignant phenotype by normal cells following the transfer of DNA from malignant human tumors. Activated oncogenes of the ras family were identified by this technique with transfection of human DNA into mouse NIH 3T3 cells.

##### Oncogene Mutations in Lung Cancer

Activation of the K-ras oncogene occurs in human NSCLC (Santos et al., 1989; Shimizu et al., 1983). Recent studies using the polymerase chain reaction (PCR) and specific oligonucleotide hybridization show that a third of NSCLC patients have ras family mutations (Rodenhuis et al., 1987; Rodenhuis et al., 1988).

However, Reynolds and coworkers, using a sensitive NIH 3T3 cotransfection-nude mouse tumorigenicity assay, found that 12 of 14 (86%) lung tumor DNAs from smokers contained activated proto-oncogenes related to the ras family (Reynolds et al., 1991). K-ras mutations occur primarily in adenocarcinomas, and the K-ras proto-oncogene has a point mutation in 30% to 40% of adenocarcinomas of the lung (Rodenhuis et al., 1987; Rodenhuis et al., 1988). Thus, a minimum of 32,000 patients per year are expected to develop ras-mutation-positive lung cancer. K-ras mutations are associated with a history of tobacco consumption and may contribute to tumor progression.

The p53 gene is the most frequently mutated gene yet identified in human cancers. It is mutated in over 50% of human NSCLC (Hollestein et al., 1991). The p53 gene encodes a 375-amino-acid phosphoprotein that can form

complexes with host proteins such as large-T antigen and E1B (Lane et al., 1990). Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. The wildtype p53 gene may directly suppress uncontrolled cell growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild-type p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene. Mutations of p53 are common in a wide spectrum of tumors (Bressac et al., 1990; Dolcetti et al., 1990; Rodrigues et al., 1990; Nigro et al., 1989); they occur in both NSCLC and SCLC cell lines and fresh tumors (Nigro et al., 1989; Takahashi et al., 1989).

Options for specific targeting of oncogenes include inhibition of expression of a dominant gene or replacement of a deleted or mutated tumor suppressor gene. Progress in the understanding of the critical genes involved in tumor development and in technology for altering gene expression logically led to our studies of techniques for achieving these options. Initially, a model for specific inhibition of K-ras was developed. We chose to work with K-ras because of the applicability of the findings to a large number of tumors, because of our previous work with K-ras, and because information on the genetic organization and sequence of the ras gene family was readily available. Advances in antisense and retroviral gene transfer technology suggested that application of these techniques may mediate specific inhibition of oncogene expression.

Antisense mRNA, which is precisely complementary to the corresponding sense mRNA, inhibits translation. The mechanisms for this inhibition have not been completely defined but include inhibition of translation by ribosomes, degradation of sense-antisense duplexes by enzymes, and failure of export from the nucleus. Thus, specific targeting of a gene in a multigene family could occur if it possessed unique sequences in a region amenable to antisense inhibition, such as an initiation codon or splice site.

The working hypothesis that was developed by the inventors is that reversal of a single altered genetic event in the cancer cell can potentially reverse critical features of the malignant phenotype of that cell. This finding has important therapeutic implications. Cancer cells have multiple genetic alterations. Therapy directed toward oncogenes will be practical only if therapeutic effects occur with targeting of one or two genes. It is unlikely that any therapy targeting oncogenes or their products will be absolutely specific for cancer cells. If other genes can compensate for loss of normal function by a specific oncogene mediated by an antisense construct, the harmful effects of the therapy will be reduced.

Studies from the inventors' laboratory indicate that reversal of a single genetic alteration has profound effects on the growth and tumorigenicity of lung cancer cells (Mukopadhyay et al., 1990; Mukopadhyay et al., 1991). Additional support for this concept comes from a recent study by Soriano and coworkers (Soriano et al., 1991) in which transgenic mice were created that lacked a functional c-src proto-oncogene. The resulting developmental defect in the mice was osteopetrosis. The ubiquity of c-src, its high degree of conservation among species, and its role in mitosis suggest that inactivation would be lethal, but this was not the case; viable mice were recovered. A possible explanation is that other closely related nonreceptor tyrosine kinases such as yes and fyn can compensate for loss of c-src. Introduction of a single copy of a wildtype tumor suppressor gene into normal cells would be unlikely to have adverse effects if it

occurred during therapy directed at replacing inactivated tumor suppressor genes in cancer cells.

Preliminary data on transfection of an antisense K-ras expression vector indicated that inhibition of expression of a single oncogene reduced the growth rate of cancer cells and tumorigenicity in nu/nu mice. However, transfected cells retained viability, as did cells with no endogenous K-ras mutation that were also transfected with the construct. The wtp53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene (Mukhopadhyay et al., 1991; Chen et al., 1990). Normal expression of the transfected wtp53 does not affect the growth of cells with endogenous wtp53. Thus, such constructs might be taken up by normal cells without adverse effects.

#### Treatment Protocol Development

The inventors have developed a protocol for the treatment of tumors susceptible to either wtp53 or antisense K-ras gene therapy. This protocol focuses regional delivery of the two gene constructs, antisense K-ras and wtp53, to lung cancer cells in patients with unresectable obstructing endobronchial cancers. The efficiency of delivery and gene expression will be evaluated both in lung cancer cells and in normal cells in vivo. This is of importance for the design of constructs that may be useful therapeutically. The effects of these constructs on clinical progression of the cancer will be studied.

It is proposed that these approaches will lead to cancer therapy based on direct alteration of gene expression in cancer cells. Current therapy relies on attempts to kill or remove the last cancer cell. However, tumor cell dormancy is an established phenomenon making effective killing highly unlikely. Although inhibition of expression of some oncogenes may be lethal to the cancer cell, in some cases cell replication will slow or cease, thus rendering these cancers clinically dormant. Even if absolute specificity is not achieved, single oncogenes may still be important targets, because it is likely that adverse effects to normal cells will be minimal.

#### Natural history of locally unresectable NSCLC

Patients with NSCLC will die of their cancer in 86% of cases. Regional delivery of gene constructs to areas at risk for development of cancer has important implications for both prevention and therapy. Failure of therapy at the primary tumor site is a significant problem (Humphrey et al., 1990; Perez et al., 1990). Of the 161,000 patients newly diagnosed with lung cancer in 1991, 45,080 will undergo surgical resection. Local recurrence as the first site of failure will occur in 9,000 of those patients. Of the remaining patients, 52% will have localized tumors. However, 38% of these patients will have local failures following radiation therapy (22,900). Thus, 31,900 patients per year could benefit from improved local-regional therapy. Patients with unresectable obstructing NSCLC that is resistant to radiation therapy or who have coexisting metastases have a median survival of 6 months or less (Komaki et al., 1992).

#### Measure of disease activity

The ultimate goal of this therapy is to halt or reverse the manifestations of the disease. The efficacy of therapy in this group of patients will be measured by determining length of patient survival, length of time the affected lobe of the lung remains aerated, and reduction in measurable endobronchial tumor. There is no curative therapy for this stage of disease and thus the outcome is predictable enough to allow for an assessment of the results of gene therapy.

#### Anticipated effect of protocol treatment

It is anticipated that the uptake of the retroviral constructs by proliferating NSCLC cells will decrease the rate of proliferation of these cells. This would increase the length of time the affected lung would remain expanded, prevent regrowth of the endobronchial tumor, and prolong the patient's survival.

#### Alternative therapies

Patients with unresectable endobronchial tumor recurrence that is partially or completely obstructing the airway and that have failed or are unable to receive external beam radiotherapy will be considered for this protocol. Existing therapies for this condition offer only short-term palliation. Most patients have recurred despite external beam radiotherapy. It may be possible to insert a brachytherapy catheter and administer additional radiotherapy. Patients receiving this treatment have a median survival of 6 months (Komaki et al., 1992). Patients failing brachytherapy would also be eligible to receive gene therapy. Tumor can be removed from the airway with the laser or biopsy forceps. This can be done in conjunction with injection of the retroviral construct thus decreasing the volume that must be injected. The administration of the retroviral constructs would not preclude the patient from receiving other palliative therapy if the tumor progresses.

#### Antisense Embodiments

As noted above, where one contemplates employing an antisense approach to selectively inhibit one of a family of genes, it will be particularly advantageous to include within the construct regions encoding an antisense intron region complementary to an intron unique to the target transcript. In such circumstances, the present invention will be generally applicable to the down-regulation of any gene which comprises a distinct intron region, particularly those oncogenes which are members of family wherein one desires to leave unaltered the expression of other family members.

The present invention will have particular application to the selective inhibition of ras gene expression. For example, in the case of ras gene tumorigenesis, only one of the various ras gene family members undergoes mutation-based protooncogene activation. The remaining, non-activated ras gene family member(s) serve useful cellular biological functions and are apparently required for normal cellular function. Thus, it is desirable to specifically down-regulate the activated ras gene product, while leaving essentially unaffected, the non-activated ras gene counterparts. Thus, the present invention will have a particular application in the context of preferentially controlling ras gene expressing.

While this aspect of the invention is exemplified in terms of the control of ras gene expression, there is, of course, no reason why the present invention will not be similarly applicable to other genes and gene families, in light of the disclosure herein and the general knowledge and skill in the art.

Generally speaking, to practice the antisense/intron aspects of the present invention in the context of the ras gene system it will be first important to determine which of the various ras genes is involved in the oncogenic process to be retarded. This is a fairly straightforward undertaking, and involves generally that one first obtain cells which are expressing the activated ras gene product. To determine the nature of the activation, one then simply extracts DNA, amplifies the specific sequences of interest (see Table 1 below), and shows the presence or absence of the mutation by either direct sequence analysis or specific hybridization with a known oligonucleotide sequence.

After the particular activated ras gene has been identified, an appropriate intron region is then selected for constructing the antisense construct. The most appropriate introns are those which have little or no homology to other known genes. In general, it will be preferable to identify an appropriate intron structure for use in connection with the present invention an analysis of the nucleic sequence of the intron, and comparison with selected that of introns of other family members or related genes. The best choice of introns will be those having 1) a different length from corresponding introns and similar location in other members of the gene family, and 2) little or no sequence homology with the introns of the other members.

An alternative, and sometimes simpler method to identify distinct introns involves a comparison of sequence homologies can be ascertained by cross-hybridization of introns from one family member with those of other genes.

In any event, representative methods for cloning ras genes corresponding to the N-ras, K-ras and H-ras genes, have been described in the literature (McGrath, et al., 1983; Shimizu, et al., 1983; Yamamoto, et al., 1985; Kraus, et al., 1984). These teachings should provide those of skill in the art with adequate direction where one seeks to obtain sequences corresponding to the various ras gene intron.

A preferred method for cloning intron sequences is through the application of PCR-amplified cloning. In this relatively well known technique, one employs oligonucleotide primers which allow the specific amplification of the desired intron region. The primer itself corresponds to exon sequences, in that these sequences will most likely be generally available in the scientific literature for the particular application envisioned. Of course, where the intron sequences are known, computer assisted comparisons may be carried out to identify distinct regions, and appropriate PCR primers designed accordingly.

Recombinant clones which incorporate intron DNA are readily achieved through the PCR amplification of the distinct desired region using primers, e.g., that border the region, incorporating the amplified DNA into a recombinant clone, and selecting recombinant clones which have received the intron DNA-bearing clones. The intron DNA containing clones are then purified, and, preferably, the cloned DNA sequenced sufficiently to ensure that it contains the desired sequences.

Intron DNA is then removed from the vector employed for intron DNA cloning, and employed in the construction of appropriate antisense vectors. This will entail, of course, placing the intron DNA in an antisense direction behind an appropriate promoter and positioned so as to bring the expression of the antisense intron under control of the promoter.

When selecting primers for intron sequence amplification, one will typically desire to employ primers such that at least 50 and preferably 100-200, nucleotides of the intron are amplified and thereby cloned. In general, it is believed that the larger the distinct antisense intron region is, the better able it will be to selectively down-regulate the targeted gene. Furthermore, it is believed that particular advantages will be realized through the selection of intron regions which include intron/exon boundaries, or simply just the intron side of the intron/exon boundaries. The reason for this is that RNA processing takes place at the intron/exon boundary of the RNA and it is believed that the antisense intron DNA will have its greatest effect when targeted to this junction.

The particular vector which one employs for introduction of antisense intron coding sequences is not believed to be

particularly crucial to the practice of the present invention, so long as the vector is capable of introducing the nucleic acid coding sequences into the genome of the targeted cell in a relatively stable fashion. By way of illustration, but not limitation, one can mention the following vectors, including N2A, LN, LNSX, Adenovirus and Adeno-associated virus.

The most preferred vector construct for targeting cells is the LNSX retroviral vector. This vector is based on the N2 vector, which contains the extended packaging signal that allows for the production of the vector at a high titer. This vector was modified by inserting a stop codon in place of the Pr65 gag start codon to prevent synthesis of Pr 65 gag, and by replacing the upstream region of the vector with the homologous region from Moloney murine sarcoma virus. These alterations prevent synthesis of viral proteins from the vector. Splicing is not required for efficient neo-protein expression. The neo gene is expressed from the upstream LTR promoter.

The following examples are included to provide actual working protocols which the inventors have developed or adopted for carrying out preferred embodiments of the invention. Those of skill in the art will readily appreciate that many of the techniques employed in the following examples are illustrative of standard laboratory practices, which have been found by the inventors to work well in the practice of the invention. It will, however, be apparent to those of skill in the art, in light of the following examples, that numerous materials and/or modifications and procedures and nevertheless achieve a useful result.

#### EXAMPLE I

##### Specific Inhibition of K-ras Expression and Tumorigenicity of Lung Cancer Cells by Antisense RNA

###### A. Introduction

A wide spectrum of human cancers harbor ras genes activated by a single point mutation (Barbacid, 1987; Rodenhuis, et al., 1987; Bos, 1989; Rodenhuis, et al., 1990; Mabry, et al., 1988; Santos, et al., 1984; Taya, et al., 1984; Cline, et al., 1987; Feig, et al., 1984; Vogelstein, et al., 1988; Kumar, et al., 1990). Despite considerable knowledge of the structural aspects of the ras gene product, the functional role in physiological and pathological processes remains elusive (Barbacid, 1987). Cellular location and structural and biochemical similarities to G proteins suggest that ras gene products are involved in signal transduction (Bos, et al., 1987; Hurley, et al., 1984). The present example describes the preparation and use of an antisense RNA construct to block selectively the production of the mutated protein in the human non-small cell lung cancer (NSCLC) cell line NCI-H460A. The direct contribution of the mutated p21 protein to the malignant phenotype was also examined.

###### B. Materials and Methods

H460, H322, H226, H522 non-small cell lung cancer (NSCLC) cell lines were generously provided by Drs. J. D. Minna, A. F. Gazdar, NCI Naval Medical Oncology Branch, Bethesda, Md. All cell lines were grown in regular RPMI medium, 5% FCS, in routine culture.

###### 1. Plasmid Construction

A 2-kb genomic DNA fragment from the K-ras proto-oncogene was subcloned into an Apr-1-neo vector in both sense and antisense orientation. A 2-kb Eco RI/Pst I fragment containing second and third exon sequences together with adjoining flanking intron sequences was isolated from the SP6 vector (Oncogene Sciences) and Klenow enzyme was used to make blunt ends. Apr-1-neo vector was digested with Bam HI and blunt end ligation was performed to obtain the Apr-1-neo AS or Apr-1-neo A constructs.

###### 2. DNA Transfections

H460a or H322a cells were electroporated with 10 ug of Apr-1-neo AS or Apr-1-neo S plasmid DNA. Forty-eight hours after transfection G418 was added into the medium at a concentration of 300  $\mu$ g/ml for H460a and 200  $\mu$ g/ml for H322a. Individual colonies were picked up and grown in culture for further analysis.

###### 3. Southern blot analysis

High molecular weight DNA was isolated and digested with Eco RI (Boehringer-Mannheim) (20  $\mu$ g), and electrophoresed in 0.8% agarose gel, transferred onto a Gene Screen membrane (NEN) and hybridized with a  $P^{32}$  nick translated 2 kb genomic K-ras DNA probe.

###### 4. Measurement of RNA Expression

Total cellular RNA was isolated from the cell lines (Chomczynsky, et al., 1987). Twenty microgram of total RNA was size fractionated in MOPS/formaldehyde gel, transferred onto a Gene Screen membrane and processed for hybridization with riboprobes. A 302 bp genomic DNA of the K-ras gene was amplified by PCR spanning the third exon and intron sequences and was subcloned into a blue-script vector. In vitro S and AS RNA probes were synthesized using either a T7 or T3 promotor.

###### 5. Polymerase Chain Reaction

Polymerase chain reactions were performed as previously described using Taq 1 DNA polymerase (Saiki, et al., 1985). Oligonucleotide primers corresponding to region the 5' and 3' regions of codons 12 and 61 of human K-ras, H-ras, and N-ras genes were synthesized. Two micrograms of genomic DNA was subjected to 35 cycles of amplification. DNA sequences of oligonucleotide primers used for PCR amplification are listed below in Table 1.

TABLE 1

Primers	Sequence	Target
KA51	5' TTC CTA CAG GAA GCA AGT AGT A 3'	K-ras 2nd exon
KB61	5' ACA CAA AGA AAG CCC DCC CCA 3'	
KA12	5' GAC TGA ATA TAA SCT TGT GG 3'	K-ras 1st & 2nd exon
KB61	5' ACA CAA AGA AAG CCC DCC CCA 3'	
HA12	5' GAC GGA ATA TAA GCT GGT GG 3'	H-ras 1st & 2nd exon

TABLE 1-continued

Primers	Sequence	Target
HB61	5' CGC ATG TAC TGG TCC CGC AT 3'	
NA12	5' GSC TGA GTA CAA ACT GGT GG 3'	N-ras 1st & 2nd exon
NB61	5' ATA CAC AGA GGA AGC CTT CG 3'	

#### 6. Slot Blot Oligonucleotide Hybridization

PCR amplified DNA samples (12.5, 25, 50 ng) were blotted onto a Gene Screen membrane using a slot blot apparatus (Schleicher & Schuell). The filters were prehybridized and hybridized at 55° C. in 6× SSC, 5× Denhardt's and 100 µg/ml of salmon sperm DNA for 2 h. Filters were washed twice in 6× SSPE at room temperature and once for 30 mins at 58° C. Finally, blots were washed for 5 mins at 64° C. The filters were exposed to x-ray film for 12–24 h at –80° C.

#### 7. Direct sequencing of PCR Amplified DNAs

PCR DNA corresponding to the second exon was purified in 8% polyacrylamide gel. A single DNA band was excised and purified DNA was used for asymmetric amplification in 100 µl of PCR reaction mixture. One (KA 61) amplicon was added to this mixture. After 20 cycles, single-stranded DNA was purified through gene clean (Bio 101) and DNA was eluted in 15 µl of water. Four microliters of DNA were mixed with 4 µl of 10× Taq 1 buffer and 1 µl (10 pmol) of a second amplicon (KB 61) was used as a sequencing primer and DNA was sequenced using a Sequenase kit.

#### 8. RNA PCR Analysis

cDNA synthesis was carried out in a total volume of 20 µl containing 5 µg of total RNA and oligo (dT) as a primer (Becker-Andre, et al., 1989). A portion of the cDNA corresponding to the first and second exons was amplified to monitor the level of endogenous K-ras mRNA (FIG. 2A) using KA12 and KB61 amplicons. Denaturation, annealing, and extension were done at 92° C. for 1 min, 51° C. for 1 min and 74° C. for 1 min, respectively. However, annealing temperatures for N-ras and H-ras were 44° C. and 42° C., respectively. In addition, two amplicons were also used in the same reaction mixture to amplify a 118-bp fragment of the p53 gene as an internal control. PCR products were either transferred onto a membrane and hybridized with <sup>32</sup>P labelled cDNA probe or alternatively, there were directly labelled during the last cycle of amplification by adding 1 uCi of <sup>32</sup>P dCTP. The labelled PCR products were loaded on an 8% nondenaturing polyacrylamide gel. The gel was photographed after ethidium bromide staining, dried, and exposed to x-ray film overnight at –80° C.

#### 9. Western blot analysis of RAS protein

Protein extracts were prepared by lysing cell in TBS (10 mM TRIS ph 7.5, 100 mM NaCl, 1 mM PMSF 1% NP40, 1% deoxycholate). The extracts were cleaned by centrifugation at 10,000× g for 1 h. The protein concentration of the supernatant was calculated spectrophotometrically. Five hundred micrograms of protein were size fractionated in 12.5% SDS polyacrylamide gel and electroblotted onto nitrocellulose membranes. Ras specific p21 protein was detected using either K-ras or pan ras specific monoclonal antibody (Oncogene Sciences) followed by <sup>125</sup>I-labelled goat anti-mouse second antibody.

#### 10. Tumorigenicity in Nude Mice

The tumorigenicity of these cell lines was examined by subcutaneous inoculation of 10<sup>5</sup> (FIG. 3B) and 10<sup>6</sup> cells in nu/nu mice. Each cell line was injected into 5 animals. Tumors were measured with linear calipers in 2 orthogonal directions by the same observer.

#### C. Results and Discussion

Segments of the K-ras gene containing first and second exons were amplified from a number of NSCLC cell line DNAs by polymerase chain reaction (Saiki, et al, 1985) and subsequently hybridized with a set of <sup>32</sup>P-labelled oligonucleotide probes (FIG. 1A-1, FIG. 2A, FIG. 2B, FIG. 2C and FIG. 2D). Mutations were confirmed by a direct PCR DNA sequencing method. A homozygous mutation at codon 61 was detected in the NCI-H460A large cell undifferentiated NSCLC cell line with a normal glutamine residue (CAA) substituted by histidine (CAT). This cell line is highly tumorigenic in nude mice.

A recombinant plasmid clone was constructed using a wildtype 2 kb K-ras genomic DNA segment carrying second and third exons together with flanking intron sequences subcloned into an Apr-1-neo expression vector (Gunning, et al., 1984) in the antisense orientation (AS; FIG. 1G). Sense orientation (S) plasmid constructs were used as a control (FIG. 2B). AS or S K-ras RNA synthesis was accomplished by transfecting H460a cells, a cloned derivative of the NCI-H460A cell line, with Apr-1-neo AS or Apr-1-neo S constructs by electroporation. The β-actin promoter of the vector was constitutively capable of directing the synthesis of RNA from the inserted DNA. The Apr-1-neo vector offered suitable G418 marker gene expression for selection of the transfectants.

Individual G418 resistant colonies were selected and grown in culture for further analysis. Stable integration of the plasmid DNA in the transfectants was examined by Southern hybridization with a 2 kb DNA insert from the original plasmid clone as a probe (FIG. 1C). The southern blot analysis showed a single 3 kb Eco RI band corresponding to the endogenous K-ras gene in the parental H460a cell line, but additional bands were observed in the individual clones indicating single or multiple copy inserts.

The extent of stable AS RNA expression and its effect on the endogenous K-ras mRNA level was investigated. Total RNA was extracted from subconfluent, growing cultures (Gunning, et al., 1987). The presence of AS and S RNA was detected by northern blot hybridization using either an S or AS RNA probe synthesized in vitro from a Bluescript vector carrying a 302 bp K-ras DNA insert corresponding to the third exon and part of the intron sequences (FIG. 1D). Interestingly, the clones carrying the Apr-1-neo AS vector show one RNA band at about 1.5 kb, but the cells carrying the S construct show two RNA species. The reason for this is unknown, but the possibility exists that the RNA synthesized from the genomic DNA under control of the β-actin

promoter could be processed *in vivo*. However, no corresponding hybridization band was detected in H460a cells, which indicated a significantly higher level of K-ras RNA was synthesized under the  $\beta$ -actin promoter.

Next, the p21 protein level in these transfectants was analyzed by western blot analysis (FIG. 1E and FIG. 1F). A K-ras-specific p21 monoclonal antibody (Oncogene Science) was used to determine the level of K-ras protein in transfectants, parental H460a cells, and Calu-1 cells, which have a high level of K-ras gene expression (FIG. 1E). Western blot analysis showed a 95% reduction in K-ras p21 protein synthesis in the clones expressing the AS RNA, while parental cells, S K-ras clones, and Calu-1 cells showed a significant level of K-ras p21 protein. These results indicate that AS RNA can effectively block the synthesis of K-ras specific protein. Since members of the ras gene family share a great deal of sequence homology and code for a similar p21 ras protein, we examined the total ras protein product in these clones was examined using a PAN ras monoclonal antibody (New England Nuclear) to determine whether a reduced level of K-ras protein reflects any change in H-ras and N-ras p21 protein synthesis (FIG. 1F). Western blot analysis revealed only a slight decrease in overall ras protein level in all clones containing Apr-1-neo-AS, as compared to 460a parental cells.

The effect of AS RNA on the specific production of mature endogenous K-ras mRNA was analyzed by cDNA PCR (FIG. 2A). cDNA synthesized from the total RNA (Chomczynsky, et al., 1987) was subjected to PCR amplification using amplimers corresponding to the 5'-end of the first exon and the 3'-end of the second exon (FIG. 2A). Because the AS RNA was generated only from a second and third exon of the K-ras gene, PCR amplified cDNA represented the level of endogenous K-ras mRNA. A 246-bp amplified DNA fragment was labelled by  $^{32}$ P dCTP and subsequently analyzed by polyacrylamide gel electrophoresis. In addition, a 118-bp segment of endogenous p53 cDNA was co-amplified in the same reaction mixture using p53 specific amplimers to serve as an internal control for the PCR.

Results showed that H460a cells, clones expressing S RNA, and the Calu-1 cell line expressed K-ras mRNA, as evidenced by the presence of a high level of amplification of the 246-bp cDNA product (FIG. 2B). H460a clones expressing AS RNA showed very little amplification, and cellular K-ras mRNA synthesis appeared to be completely inhibited (FIG. 2B, lanes 5 and 6). In contrast, the endogenous p53 expression remained unaffected. This prompted us to investigate the level of expression for other ras genes in these clones. We employed the same cDNA PCR methodology to analyze the N-ras and H-ras mRNA level using N-ras and H-ras-specific oligonucleotides as amplimers. A steady state level of H-ras and N-ras gene expression was observed, but no obvious change either in Apr-1-neo AS or Apr-1-neo S transfectants was noticed (FIG. 2C and FIG. 2D). The p53 gene expression serving as a control in these experiments remained unaffected. Thus, inhibition of K-ras expression by our AS RNA construct is specific.

H460a clones expressing AS K-ras RNA continued to grow in culture. However, H460a Apr-1-neo AS transfectants showed a three-fold reduction in growth, compared to the H460a Apr-1-neo-S transfectants and the parental H460a cells (FIG. 3A). The H322 NSCLC cell lung cancer cell line has wild-type ras family genes. H322 Apr-1-neo AS and Apr-1-neo S transfectants had identical growth characteristics, indicating that inhibition of wild-type K-ras is not sufficient to alter tumor cell growth rate. These results

together indicate that the presence of sense K-ras RNA did not alter the growth kinetics of H460a cells. However, the marked growth retardation of the K-ras Apr-1-neo-AS transfectants suggests that the mutated p21 protein contributes to the faster growth rate of these cells.

The tumorigenicity of cell lines expressing AS RNA was assessed by subcutaneous injection of  $10^5$  and  $10^6$  cells in nu/nu mice. Subcutaneous inoculation of H460a cells at both doses led to the formation of tumors in 15 days in all mice (3 to 5 mice per group in 3 separate experiments). No tumor developed in mice injected with  $10^5$  cells for both clones of H460a AS cells during 120 days of observation in a total of ten mice, whereas all mice receiving H460a cells developed tumors. When the inoculum was increased to  $10^6$  cells, tumors grew in all mice, but the tumors in mice receiving AS clones grew at a slower rate than H460a cells or the S control (FIG. 3B). Tumors were excised and analyzed for K-ras expression by cDNA-PCR. K-ras expression was not detected in tumors arising from injection of AS clones but was present in S clones and H460a tumors.

The above experiments indicate that in H460a cells engineered to synthesize AS K-ras RNA, the level of K-ras mRNA and K-ras p21 protein are effectively down regulated. Reduction in the expression of K-ras mutated gene reproducibly reduced the rate of tumor growth in nu/nu mice. Our studies show that a construct can be made that distinguishes among members of the ras family. Previous studies with AS oligonucleotides showed inhibition of p21 expression which led to cell death (Brown, et al., 1989; Debus, et al., 1990). Our data indicate that AS RNA generated from the genomic DNA of the K-ras gene can specifically inhibit K-ras expression. In our model inhibition of activated K-ras reduced the growth rate of the H460a cells. However, there was no effect on cell viability or continued growth in culture. This suggests that redundancy in p21 expression may compensate for absence of expression by one member of this family so that functions essential for maintenance of cell viability are preserved. However, tumorigenicity was maintained in the absence of activated K-ras expression although the rate of tumor growth was diminished. We hypothesize that in human NSCLC, ras mutations confer a growth advantage to the malignant cell.

## EXAMPLE II

### Retroviral Vector-mediated Transduction of K-ras Antisense RNA Into Human Lung Cancer Cells Inhibits Expression of the Malignant Phenotype

In overview, the present example illustrates a retroviral vector system that was developed by the inventors to efficiently transduce K-ras antisense constructs into human cancer cells. The 1.8-Kb K-ras gene fragment DNA in antisense (AS) orientation to a  $\beta$ -actin promoter was inserted into retroviral vector LNSX in two different orientations. The constructs were transduced into amphotropic packaging cell line GP+envAm12 followed by alternating infection between the ecotropic packaging cell line  $\Psi$  2 and GP+envAm12. Titers up to  $9 \times 10^6$  CFU/ml were achieved without detectable replication-competent virus being produced. The human large cell lung carcinoma cell line H460a, which has a homozygous codon 61 K-ras mutation, was transduced, and a transduction efficiency of 95% was obtained after 5 to 7 repeated infections.

DNA polymerase chain reaction analysis showed that the retroviral construct was integrated into the genome of H460a cells. K-ras antisense RNA expression was detected in the



cells by slot blot hybridization with a specific oligonucleotide probe. Translation of the mutated K-ras p21 protein RNA was specifically inhibited, whereas expression of other p21 species was unchanged. Proliferation of H460a cells was suppressed tenfold following transduction by LNSX-AS-K-ras. Colony formation in soft agarose and tumorigenicity in an orthotopic nu/nu mouse model were dramatically decreased in H460a cells expressing antisense K-ras.

#### A. Materials and Methods

##### 1. Cells and Culture Conditions

NIH-3T3 cells, the human fibroblast cell line MRC-9, and ecotropic retrovirus packaging cell line  $\Psi$ 2 (Mann et al., 1983) were grown in Dulbecco-modified Eagle's Medium (DMEM; GIBCO) with a high glucose content (4.5 g/l) supplemented with 10% fetal bovine serum (Sigma Chemical Co.). The amphotropic retrovirus packaging cell line GP+envAm12 [(Markowitz et al., 1988); a gift from Dr. Arthur Bank] was grown in DMEM with high glucose; 10% newborn calf serum; 15  $\mu$ g/ml hypoxanthine, 250  $\mu$ g/ml xanthine, and 25  $\mu$ g/ml mycophenolic acid (HXM medium); and 200  $\mu$ g/ml hygromycin B (Sigma Chemical Co.). Non-small cell lung cancer cell (NSCLC) line H460a was maintained in RPMI 1640 medium with 5% fetal bovine serum (Mukhopadhyay et al., 1991). All cells were also supplemented with 2 mM L-glutamine and antibiotics. The H460a was established in culture from a human large cell undifferentiated non-small cell lung cancer. This cell line has a homozygous codon 61 K-ras mutation (Mukhopadhyay et al., 1991). The MRC-9 cell line has no evidence of mutations at codon 12 or 61 of the K-ras gene by single strand conformation polymorphism (SSCP) analysis and chain termination sequencing.

##### 2. Retroviral Vector Construction

Retroviral vector LNSX contains the selectable neo gene and a unique cloning site for cDNA insertion. The neo gene is expressed from the retroviral long-term repeat (LTR), and the inserted gene has the simian virus 40 (SV40 early promoter (Miller et al., 1989). A recombinant plasmid clone was constructed using a wild-type 2-Kb genomic DNA segment carrying second and third exons together with flanking intron sequences subcloned into an Apr-1-neo expression vector in the antisense orientation with a  $\beta$ -actin promoter (Mukhopadhyay et al., 1991). The 5.8-Kb EcoR I/Nde I fragment of  $\beta$ -actin K-ras antisense was isolated from this plasmid, and Klenow enzyme was used to blunt the ends. To obtain the recombinant constructs in two different orientations (a and b) relative to the SV40 promoter (FIG. 4A), the LNSX retroviral vector was digested with Stu I (orientation a) or Hind III (orientation b) and blunt end ligation or Hind III linker ligation was performed. *E. coli* bacteria were transformed by this recombinant plasmid DNA, and clones were screened by enzyme analysis. Southern hybridization with the 1.8-Kb  $^{32}$ P-nick-translated genomic K-ras DNA fragment probe was used to confirm the construction of the positive clones using the following hybridization condition: 6 $\times$  SSC, 10 $\times$  Denhart's solution, 0.1% sodium dodecyl sulfate (SDS), 100  $\mu$ g/ml salmon sperm DNA, and 25 mM  $\text{NaH}_2\text{PO}_4$  for 2.5 h at 65 $^\circ$  C.

##### 3. Virus Production and Infection Efficiency

Amphotropic packaging cell line GP+envAm12 was transfected with recombinant  $\beta$ -actin K-ras antisense LNSX plasmid DNA by the calcium phosphate co-precipitation method (Graham et al., 1973). Forty-eight hours later, the transfected cells were placed in medium containing G418 (400  $\mu$ g/ml). Colonies of "producer cells" were selected 10–14 d later and expanded into large cultures.

The viral titer was tested by infecting NIH-3T3 cells. Plates (60 mm) were each seeded with  $5 \times 10^5$  NIH-3T3 cells. After 24 h, the medium on these plates was replaced with 1 ml of serial dilutions of medium conditioned for 24 h by confluent cultures of producer cells. Polybrene was added to a final concentration of 8  $\mu$ g/ml. The cells were incubated 2–4 h and then 4 ml of fresh medium was added. Forty-eight h after the infection, the infected cells were trypsinized and replated onto 100-mm tissue-culture dishes in medium containing 400  $\mu$ g/ml G418. Colonies could be counted 10–14 d later.

The high-titer GP+envAm12 cells transfected by  $\beta$ -actin K-ras antisense LNSX (orientation a) were mixed with ecotropic packaging cell line  $\Psi$ 2 at a ratio of 1:1. A total of  $5 \times 10^5$  cells from this mixture was seeded onto 100-mm plates and passaged continuously for 1 month. These cells were then selected by HXM medium (containing 200  $\mu$ g/ml hygromycin B and 400  $\mu$ g/ml G418) for 10–14 d. The amplification of retrovirus production was tested by infecting NIH-3T3 cells. Supernatants from NIH-3T3 cells infected by GP+envAm12-producing cells and selected with 400  $\mu$ g/ml G418 for 10–14 d (short-term assay) or passaged continuously for 1 month without G418 selection (long-term assay) were used to infect fresh NIH-3T3 cells to detect the existence of replication-competent retrovirus.

NSCLC cell line H460a was infected once by incubating  $10^5$  cells in 6-well plates with 1 ml of each serial dilution (1:1, 1:10, 1:100, 1:1000) of recombinant LNSX-antisense (orientation a) retroviral stock in the presence of 8  $\mu$ g/ml polybrene. In another assay,  $10^4$  H460a cells were incubated with 0.5 ml LNSX-antisense (orientation a) retroviral stock (virus titer:  $2 \times 10^6$  CFU/ml) in 12-well plates, and 8  $\mu$ g/ml polybrene was added. The retroviral supernatant was added daily, following removal of medium and washing of the cultured cells, for 1–7 d. Control cultures were incubated with fresh medium. Two days after these infections were completed, equal numbers of H460a cells were seeded into a selective medium containing 300  $\mu$ g/ml G418 or nonselective medium for 10–14 d. The infection efficiency for an infected cell population was measured by dividing the number of G418-resistant colonies by the number of colonies growing in the absence of selection.

##### 4. PCR Analysis of Genomic DNA From Transduced H460a Cells

Genomic DNA was isolated by SDS-proteinase K lysis of H460a cells followed by phenol-chloroform extraction. One microgram of genomic DNA was placed in a total volume of 100  $\mu$ l containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM  $\text{MgCl}_2$ , 0.1% gelatin, 20 mM deoxyribonucleoside triphosphates, 660 ng each of two neomycin phosphotransferase (neo-r) oligonucleotide primers (neo 1: CAAGATG-GATTGCACGCAGG; neo 5: CCCGCTCAGAAGAACTCGTC), and 2.5 units of Taq DNA polymerase. The tubes were cycled 35 times through 94 $^\circ$  C. for 1 min, 50 $^\circ$  C. for 1 min, and 72 $^\circ$  C. for 2 min. The PCR products (15  $\mu$ l) were electrophoresed on 2% gel (1% agarose, 1% nusieve GTG agarose) stained with ethidium bromide. The DNA was transferred onto a nitrocellulose membrane and hybridized with  $^{32}$ P-nick translated neo gene probe (Hind III/Sma I neo gene fragment of Psv2-neo plasmid DNA) in 6 $\times$  SSC, 10 $\times$  Denhart's solution, 0.1% SDS, 100  $\mu$ g/ml salmon sperm DNA, and 25 mM  $\text{NaH}_2\text{PO}_4$  at 65 $^\circ$  C. for 3 h.

##### 5. Slot Blot Hybridization of Poly(A $^+$ ) RNA

Poly(A $^+$ ) RNA was isolated from the cell lines. The RNA was denatured with 50% formamide, 6% formaldehyde, and

1× SSC at 68° C. for 15 min, then blotted onto nitrocellulose membranes (8 µg, 4 µg, or 2 µg) using a slot blot apparatus. The filters were prehybridized and hybridized at 64° C. for 8–12 h with a <sup>32</sup>P-end-labeled 42-bp K-ras exon 2 DNA oligonucleotide probe in 1× SSPE, 2× Denhardt's solution, 1% nonfat dry milk, 10% dextran sulfate, 2% SDS, 200 µg/ml salmon sperm DNA, 200 µg/ml yeast tRNA, and 200 µg/ml polyadenylic acid. They were then washed twice in 1× SSPE, four times in 0.2× SSPE for 30 min at room temperature, and finally with 0.1× SSPE for 30 min to 1 h at 47–58° C. The filters were exposed for 2–3 d at 80° C. A β-actin probe was used to reprobe the filters to confirm equal loading of RNA.

#### 6. Immunoblot Analysis of ras Protein

Protein extracts were prepared by lysing cells in Laemmli buffer (130 Mm Tris-HCl, Ph 6.8; 2% SDS; 10% glycerol). The extracts were boiled for 5 min, cooled in ice and cleared by centrifugation at 10,000× g for 15 min. The protein concentrations were calculated by bovine serum albumin protein assay. One hundred micrograms of protein was size-fractionated by 12.5% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. ras-specific p21 protein was detected using either a K-ras or a pan-ras-specific p21 monoclonal antibody (Oncogene Science, Mahasset, N.Y.) followed by horseradish peroxidase-labeled goat anti-mouse second antibody (Pierce, Rockford, Ill.). The change in K-ras p21 levels was determined by measuring absorbance with a video densitometer (Model 620, Bio-Rad, Richmond, Calif.).

#### 7. Proliferation and Soft Agarose Colony Formation by H460a cells

Parental and infected H460a cells (10<sup>3</sup>/well) which were selected or not selected with 300 µg/ml G418 were seeded and grew in 12-well plates for 7 d. Cells were harvested and counted at different days. Human fibroblast cell line MRC-9 was used as a control. Aliquots of 5×10<sup>4</sup> cells were mixed with 0.35% agarose in RPMI 1640 medium and plated over a base layer of 0.7% agarose and culture medium hardened in 60-mm dishes. Colonies (>50 cells) were counted using a phase contrast microscope 10–14 d later.

#### 8. Tumorigenicity of H460a cells in Orthotopic Lung Cancer Model

A model of orthotopic lung cancer growth in nu/nu mice was used to measure tumorigenicity of these cells. Balb/c nu/nu mice were irradiated with 350 Cgy of whole-body irradiation from a <sup>60</sup>Co source at 127 cGy/min. After being anesthetized with methoxyflurane, the H460a-antisense-LNSX construct, the H460a cells infected by the retroviral vector alone, or H460a parental cells were injected endotracheally (10<sup>5</sup>/mouse) using a 27-gauge blunt needle. The mediastinal block was harvested after 4 wk and tumor growth was measured with linear calipers in two orthogonal directions without knowledge of the animal treatment group.

#### B. Results

##### 1. Construction and Generation of β-actin K-ras Antisense LNSX Replication-defective Retrovirus

Recombinant plasmid clones were constructed by subcloning a wild-type 1.8-Kb K-ras genomic DNA segment carrying second and third exons together with flanking intron sequences and a β-actin promoter in antisense orientation into an LNSX retrovirus vector in two orientations (FIG. 4A and FIG. 4B). The plasmid DNA was analyzed by restriction enzyme mapping with controls of LNSX plasmid DNA only and the β-actin K-ras antisense Apr-1-neo vector. β-actin K-ras antisense LNSX was constructed in two different orientations, both of which included the 4-Kb β-actin

promotor, the 1.8-Kb K-ras fragment, and a 6-Kb LNSX vector fragment. The digested DNA was transferred to a nitrocellulose membrane and hybridized with a 1.8-Kb <sup>32</sup>P-nick-translated genomic K-ras probe. Orientation a has the K-ras 5' end adjacent to the SV40 promoter of LNSX and thus is placed in a reverse orientation ((LTR\_neo\_SV40\_K-ras\_β-actin\_LTR). Orientation b has the β-actin promoter adjacent to the SV40 promoter (LTR\_neo\_SV40\_β-actin\_K-ras\_LTR).

The amphotropic retrovirus was produced by transfection of the GP+envAm12 packaging cell line with this recombinant DNA. To increase recombinant retrovirus production, amphotropic β-actin K-ras antisense LNSX (orientation a) GP+envAm12 cells were co-cultivated with ecotropic Ψ-2 for 1 month. This mixed-cell pool was selected by HXM medium with hygromycin B and G418. The highest viral titer generated by testing the selected colonies was 9×10<sup>6</sup> CFU as determined by transduction and selection of NIH-3T3 cells.

Replication-competent virus produced by GP+envAm12 was measured by infection of fresh NIH-3T3 cells with medium conditioned in NIH-3T3 cell cultures infected by recombinant retrovirus and selected by G418 for 10–14 d (short-term assay). In a more sensitive long-term assay, NIH-3T3 cells were infected with the medium conditioned by GP+envAm12-producing cells, after which they were passaged for 1 month to allow for the spread and amplification of a rare recombinant wild-type virus in the culture. Medium collected from these NIH-3T3 cells was used to infect fresh NIH-3T3 cells. Both the short-term and long-term assays showed that no detectable replication-competent retrovirus was produced by GP+envAm12 cells.

##### 2. Infection Efficiency in H460a cells

H460a cells were infected with recombinant LNSX-antisense retrovirus by incubating with viral stocks in the presence of 8 µg/ml polybrene. The infection efficiencies of H460a cells at varying virus to H460a cells ratios (V/T) of 1:10, 1:1, 10:1, and 100:1 were 7.5±1%, 26±1.2%, 53±11%, and 57±13% after a single cycle of infection (FIG. 5A). The efficiency increased with higher V/T ratios and plateaued at the 10:1 V/T ratio. The infection efficiency increased also with the number of infection exposures at the same V/T ratio (100:1) (FIG. 5B). Infection efficiencies of 97±15% and 25±2.6% were achieved after five cycles of infection of H460a cells at 10:1 and 1:10 V/T ratios, respectively.

##### 3. Detection of Transduced neo gene by PCR in Infected H460a cells

Genomic DNA was isolated and amplified by PCR with neo 1 and neo 5 oligonucleotide primers. A 790-bp segment of the neo gene was detected in transduced H460a cells, but not in parental H460a cells (FIG. 6A). Southern hybridization with a <sup>32</sup>P-nick-translated neo gene probe confirmed the identity of the neo gene band (FIG. 6B), indicating that the inserted retrovirus gene was successfully integrated into H460a genomic DNA.

##### 4. Expression of K-ras Antisense RNA and Specific Inhibition of K-ras Protein p21 in H460a cells

Poly(A<sup>+</sup>) RNA was extracted from parental and infected H460a cells. The expression of K-ras antisense RNA was detected by slot blot hybridization with a 42 bp K-ras exon 2 oligonucleotide probe (FIG. 7A and FIG. 7B). The level of expression of K-ras antisense RNA in H460a cells infected by the orientation (a) retrovirus was higher than that of H460a cells infected by the orientation (b) retrovirus. Reprobing the filter with the β-actin DNA probe showed that each sample was loaded equally.



The inventors next analyzed the p21 protein level in these H460a cells by immunoblot analysis. A K-ras-specific p21 monoclonal antibody was used to determine the level of K-ras protein in parental and infected H460a cells. K-ras p21 protein synthesis was reduced by 90% in the H460a cells expressing high levels of K-ras antisense RNA (orientation a retrovirus) and by 30% in the H460a cells infected with orientation b retrovirus, compared with those of parental H460a cells and H460a cells infected only by the LNSX vector retrovirus (FIG. 8A). The total ras protein production in these cells was also examined, using a pan-ras monoclonal antibody, to determine whether a reduced level of K-ras protein reflected any change in H-ras and N-ras p21 protein synthesis. The western blot analysis revealed that overall ras protein levels in all infected cells were only slightly decreased from the level in H460a parental cells (FIG. 8B).

#### 5. Suppression of H460a Cells' Growth in Vitro and Colony Formation in Soft Agarose

H460a cells expressing K-ras antisense RNA continued to be viable in culture. However, growth of H460a cells expressing high levels of K-ras antisense RNA (orientation a) was reduced compared with that of H460a cells infected with LNSX vector only and parental H460a cells (FIG. 9A). Transduction of the human lung fibroblast cell line MRC-9, which has a wildtype K-ras gene, with LNSX and LNSX-AS-K-ras (a) did not affect proliferation of that cell line (FIG. 9B). Previous studies have shown that expression of the 1.8-Kb K-ras fragment in the sense orientation does not affect proliferation or tumorigenicity of H460a cells (Mukhopadhyay et al., 1991).

The effect of K-ras antisense RNA expression on the growth of soft agarose colonies of H460a cell lines was determined. Colony formation in soft agarose was dramatically decreased in H460a cells expressing K-ras antisense RNA (number of colonies, orientation a:  $135 \pm 26$ ; orientation b:  $320 \pm 37$ ) as compared with parental H460a cell line ( $1096 \pm 434$ ) and H460a cells infected only with retroviral vector LNSX ( $1048 \pm 322$ ) (FIG. 10A, FIG. 10B, FIG. 10C and FIG. 10D).

#### 6. Suppression of Tumorigenicity in an Orthotopic Lung Cancer nu/nu Mouse Model

Intratracheal inoculation of H460a cells in irradiated nu/nu mice resulted in the growth of endobronchial tumors with mediastinal extension in >80% of the mice after 4 wk. Twelve of 14 mice inoculated with parental H460a cells and seven of nine mice inoculated with H460a cells infected with the LNSX vector developed tumors (Table 2). Three of seven mice inoculated with H460a cells transduced with the LNSX-AS-K-ras(b) had tumors. Cells expressing the highest level of AS-K-ras with the greatest reduction in p21 expression had the lowest incidence of tumor formation. Only three of 17 mice receiving H460a-LNSX-AS-K-ras(a) cells had tumors and the volume of these tumors was much less than tumors in the control groups. Statistical analysis (chi-square) shows that there is a statistically significant difference ( $p < 0.005$ ) in tumorigenicity between H460a-LNSX-AS-K-ras(a) and the control groups.

TABLE 2

Tumorigenicity of H460a in orthotopic nude mice model

Cell lines	Cells injected	Mice with Tumors (%)	Mean volume (mm <sup>3</sup> ) <sup>1</sup>
H460a	10 <sup>5</sup>	12/14 (86)	39.9 $\pm$ 4.25
H460a-LNSX	10 <sup>5</sup>	7/9 (78)	12.5 $\pm$ 2.2
H460a-LNSX-AS-K-ras (a)	10 <sup>5</sup>	3/17 <sup>2</sup> (18)	2.95 $\pm$ 1.25
H460a-LNSX-AS-K-ras (b)	10 <sup>5</sup>	3/7 (43)	1.74 $\pm$ 1.5

Note:

The irradiated (350 Cgy) nude mice were inoculated with 10<sup>5</sup> H460a cells intratracheally.

Tumors were measured after 30 days.

<sup>1</sup>Mean volume based only on mice with tumors.

<sup>2</sup>p < .005 compared to H460a and H460a-LNSX by  $\chi^2$  analysis

#### B. Discussion

A retroviral vector-mediated gene transfer system was developed to introduce a partial K-ras genomic sequence into lung cancer cell line H460a, which has the K-ras gene mutation at codon 61. The K-ras sequence carries second and third exons together with flanking intron sequences and a  $\beta$ -actin promoter in antisense orientation. The transduced K-ras antisense gene was integrated and efficiently expressed in H460a cells. For H460a cells expressing AS-K-ras, K-ras-specific p21 protein expression was reduced more than 90%, whereas the total ras protein production decreased only slightly relative to the control group (parental H460a cells and those infected only by the retroviral vector). Specific inhibition of oncogene (e.g., N-ras, H-ras) expression by antisense oligonucleotides has been reported by a few laboratories, but the short biological half-life and low transfer efficiency of oligonucleotides in the cell were problems in those studies (Saison-Behmoaras et al., 1991; Chang et al., 1991; Neckers et al., 1992).

In the presently disclosed retroviral gene transfer system, high transfer efficiency, prolonged expression of K-ras antisense RNA, and inhibition of K-ras p21 protein were achieved, particularly through the use of reverse orientation constructs. Cells expressing the antisense K-ras construct have been grown in continuous culture for over 6 months. The expression of the neoplastic phenotype of the H460a cell line, including growth rate, ability to form colonies in soft agarose, and tumorigenicity in nude mice, were dramatically reduced. Previous studies have shown that cancer cells often have multiple genetic alterations. Therapy directed toward oncogenes will be practical only if therapeutic effects occur with targeting of one or two genes. In this case reversal of a single genetic lesion resulted in suppression of critical features of the malignant phenotype.

The inventors results indicate that the expression of the mutated K-ras protein plays an important role in the oncogenesis and growth of cell line H460a. When human fibroblast cell line MRC-9 and NSCLC cell line H322a, which has a wildtype K-ras gene, were infected by the LNSX-antisense retrovirus, the growth curves were not significantly different from that of the control cells. Thus, this construct can distinguish among closely related members of the ras family. Continued viability of cells expressing AS-K-ras suggests that other closely related members of the ras family may subsume the function of K-ras.

It is unlikely that any therapy targeting oncogenes or their products will be absolutely specific for cancer cells. If other genes can compensate for loss of normal function by a specific oncogene mediated by an antisense construct, the

harmful effects of the therapy will be reduced. Additional support for this concept comes from a recent study by Soriano and coworkers (Soriano et al., 1991) in which transgenic mice were created that lacked a functional c-src proto-oncogene. The resulting developmental defect in the mice was osteopetrosis. The ubiquity of c-src, its high degree of conservation among species, and its role in mitosis suggest that inactivation would be lethal, but this was not the case; viable mice were recovered. A possible explanation is that other closely related nonreceptor tyrosine kinases such as yes and fyn can compensate for loss of c-src.

Efficient transfer of constructs that can modify expression of oncogenes and tumor suppressor genes is critical to the analysis of the functional role of these genes and the potential therapeutic use of these constructs. We found that infection efficiency could achieve 97% using a multiple infection protocol. After one exposure, efficiency as high as 57% was achieved at a 10:1 V/T ratio, with little additional increase in efficiency obtained by increasing the V/T ratio to 100:1, indicating that such factors as the quality of the virus preparation and the proliferation status of the H460a cells may affect the infection efficiency. In the low V/T ratio (1:10) assay, infection efficiency of about 25% was obtained after five exposures. Ratios of retrovirus to tumor cells or premalignant cells such as these are achievable with regional therapy in the setting of minimal residual cancer or premalignant conditions. This revealed that, in the clinical setting, even if the high V/T ratio cannot be achieved, a satisfactory infection efficiency may be obtained by multiple infection exposures. Not all the patient's cancer cells will be in the proliferative stage at each infection exposure, and the retrovirus may selectively infect only the proliferating cells (Miller et al., 1990). Multiple exposures to the retrovirus can address this problem and maximize the number of transduced tumor cells. The use of a promoter which is commonly expressed in epithelial cells may also contribute to efficient expression in human cancer cells of epithelial origin.

The high titer ( $9 \times 10^6$  CFU/ml) of the producer cell line was obtained by "ping-pong" infection between the amphotropic packaging cell line GP+envAm12 and ecotropic packaging cell line  $\Psi$ -2. The titer was 100 times more than those of cell lines produced by GP+envAm12 before "ping-pong" infection. A similar result was reported by Bodine et al., but in their assay all of the high-titer cell lines after ping-pong infection also produced replication-competent viruses (Bodine et al., 1990). In the present system, no detectable replication-competent virus were produced even in the stringent long-term assay. This may be due to the safety of GP+envAm12, in which the Moloney murine leukemia virus gag, pol gene, and 4070A amphotropic env gene are separated on P<sub>gag</sub>-pol gpt and P<sub>env</sub>Am, two different plasmids, and the packaging signals and 3' long-terminal repeats are removed. The three specific recombination events required to generate replication-competent viruses are unlikely to occur in this system (Markowitz et al., 1988).

A very interesting finding is that, of the two orientations of the construct in the recombinant retroviral vectors [LTR<sub>neo</sub>-SV40-K-ras- $\beta$ -actin-LTR (a) and LTR<sub>neo</sub>-SV40- $\beta$ -actin-K-ras-LTR (b)], the orientation (a) vector showed higher transfection efficiency, higher virus titer, and higher K-ras antisense RNA expression efficiency. It is possible that the SV40 promoter may suppress the  $\beta$ -actin promoter as described in other systems (Emerman et al., 1984). However, the SV40 promoter is not as active as the  $\beta$ -actin promoter, and therefore this effect may have some degree of promoter specificity (Gunning et al., 1987; Emer-

man et al., 1986). If some sense transcripts were produced by this promoter in orientation (a), the splicing of the intron sequence would render the transcripts unable to hybridize with the antisense transcripts. The effectiveness in the reduction of K-ras p21 protein by orientation (a) supports the absence of this type of inhibitory effect. Interestingly, the use of a  $\beta$ -actin promoter in orientation (b) with an LNL6 retrovirus yielded low rates of infectivity and low levels of gene expression (Owens et al., 1991).

According to the original "seed and soil" hypothesis proposed by Paget in 1889, organ-site specific implantation of tumor cells is essential for optimal growth and progression of tumors in vivo (Paget, 1889). This concept has been widely supported by numerous studies in metastatic tumor models (Fidler, 1986) and, recently, athymic nude mice models have been used to study the orthotopic propagation of selected human solid tumors, including lung cancer (Howard et al., 1991). We successfully used an intratracheal model for the orthotopic propagation of human lung cancer H460a cells in irradiated nude mice to assess the tumorigenicity of the transduced cells. The H460a cells grew well in the model, and the tumorigenicity of H460a cells expressing K-ras antisense RNA was dramatically decreased. Further studies using retroviral vectors as a regional delivery method for K-ras antisense gene expression in vivo are in progress in our laboratory. The orthotopic in vivo model in use closely resembles the clinical setting, allowing a further assessment of the feasibility of using the recombinant retrovirus therapeutically in lung cancer.

### EXAMPLE III

#### Clinical Protocol for Modification of Oncogene and Tumor Suppressor Gene Expression in Non-Small Cell Lung Cancer

This example is provided to demonstrate a protocol for administering and assessing the efficacy and toxicity of the intralesional administration of retroviral constructs containing antisense (AS) K-ras (for tumors with mutated K-ras) and wildtype p53 (wtp53) (for tumors with mutated or deleted p53) into residual endobronchial NSCLC which obstructs a bronchus and which is refractory to conventional therapy.

#### A. Downregulation of activated K-ras/expression with an antisense construct

##### 1. Gene construct

The retroviral vector construct contains the AS-K-ras fragment with its  $\beta$ -actin promoter inserted into the LNSX vector (Miller et al., 1989; Palmer et al., 1987). The orientation of the insert is such that the transcription of the AS-K-ras is driven by the  $\beta$ -actin promoter in an orientation that is reverse with respect to transcription from the retroviral LTR.

##### 2. Packaging

Because recombination events may lead to the production of a replication-competent virus, a safe and efficient amphotropic packaging cell line is necessary for transfer of exogenous genes into human cancer cells. The packaging cell line employed is constructed so the gag-pol and env genes are separated on two different plasmids (Markowitz et al., 1988). The packaging signals and 3' LTRs have also been removed; this was done by transfection of NIH 3T3 cells by a plasmid containing Moloney murine leukemia virus gag and pol genes and a separate plasmid containing the env gene. The GP+envAM12 clone that produces high levels of env protein was selected to be used as the packaging cell line. The combination of mutations for the two plasmids

requires at least three recombination events between the helper plasmids and the retroviral vector; the improbability of this sequence of events essentially eliminates the possibility of replication-competent virus production. The presence of functioning retroviral genes in the packaging cell line will be monitored by an assay for reverse transcriptase production and by immunoprecipitation of env protein by metabolic labeling and immunoprecipitation with anti-env antiserum (Markowitz et al., 1988).

Continued absence of infectious virus will be determined from transfection-infection experiments. A neo-containing vector will be transfected into GP+envAm12 cells; colonies will be selected with G418. The supernatants will be used to infect NIH 3T3 cells. Selection with G418 will be done after one month to ensure the survival of rare recombinants that do not have the neo gene but subsequently infect neo-positive cells. Supernatants from the infected NIH 3T3 cells should not be infectious. These secondary supernatants will be used to infect naive NIH 3T3 cells. Lack of infectivity will indicate absence of replication competent virus.

A protocol for generating retroviral particles is as follows:

- (1) GP+envAm12 cells are grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum, 15  $\mu\text{g/ml}$  hypoxanthine, 250  $\mu\text{g/ml}$  xanthine, and 25  $\mu\text{g/ml}$  mycophenolic acid and selected in 200  $\mu\text{g/ml}$  hygromycin.
- (2) Vectors are transfected by electroporation.
- (3) G418 (400  $\mu\text{g/ml}$ ) selection is begun 48 hr after transfection and colonies are expanded 10 to 14 days later.
- (4) The viral titer is tested by infecting NIH 3T3 cells. After producer cells are semiconfluent, medium is replaced with Dulbecco's modified Eagle's medium containing 10% newborn calf serum but without G418. Cells are seeded at  $5 \times 10^5$  in 60-mm dishes. The medium is removed 18 hr later, filtered (0.45 micron), and diluted serially ( $10^2$  to  $10^7$ ). One milliliter of medium is applied to cells. Polybrene (8  $\mu\text{g/ml}$ ) is added. Cells are incubated for 2 hr at  $37^\circ$ , and then 4 ml of fresh medium is added.
- (5) After 48 hr cells are replated onto 100 mm tissue culture dishes and selected with G418. Previous human studies have used the PA317 producer cell line. This cell line is preferred because of the extensive experience with its use and prior approval for human use.

#### 4. Preclinical studies

The 2 Kb K-ras fragment (genomic exons 2 and 3) with a  $\beta$ -actin promoter was cloned into the LNSX retroviral vectors in both orientations. The p53 cDNA with its  $\beta$ -actin promoter was cloned into the LNSX retroviral vectors in both orientations. Both the LNSX-AS-K-ras and the N2A-AS-K-ras have been successfully packaged in the GP+envAm12 packaging cell line. Initial titers ranged up to  $10^4$ . By using a "ping-pong" technique, the titer of the LNSX-AS-K-ras supernatant was increased to  $5 \times 10^6$ . In this technique, supernatants from the GP+envAm12 packaging cell line were used to transduce the ecotropic packaging cell line  $\Psi 2$  (Mann et al., 1983). Supernatants from this transduction were used again to transduce GP+envAm12. Both constructs were then transduced into H460a cells. Specific expression of K-ras AS RNA was shown by slot blot analysis using vector only negative controls and a  $\beta$ -actin probe for a loading control. Western blotting studies showed that expression of the K-ras p21 protein was specifically reduced. Next the effect of multiple cycles of transduction on transduction efficiency was assessed. Transduction effi-

ciency was assessed on a functional level (FIG. 11). H460a cells were transduced with either LNSX or LNSX-AS-K-ras daily for 4 consecutive days. Cells grew for 7 days without selection.

The percent reduction in the growth fraction of the AS transduced cells reflects the efficiency of transduction as growth of a selected population of AS transduced cells does not occur during this time period. The growth of the unselected AS transduced cells was less than 20% at 7 days. Thus, the simple manipulation of exposing cells to the packaged retrovirus for 4 consecutive days caused a striking increase in transduction efficiency. In a subsequent experiment H460a cells were transduced daily for 7 consecutive days with LNSX-AS-K-ras and then selected for colony formation in G418 (FIG. 12). Colonies were compared to H460a cells that were not exposed to selective medium. Following selection the efficiency of colony formation by the transduced cells was 98%. This reinfection strategy is applicable to regional therapy. The apparent low toxicity of the retroviral constructs should permit multiple treatments. It is anticipated that the residual number of endobronchial tumor cells can be reduced to  $<10^7$  so that an excess ratio of retroviral particles to proliferating tumor cells can be achieved.

The tumorigenicity of the transduced H460a cells was studied in an orthotopic lung cancer model. Intratracheal inoculation of H460a cells in irradiated (350 cGy) nu/nu mice resulted in the growth of endobronchial tumors with mediastinal extension in  $>80\%$  of mice after 4 weeks. The H460a-AS-LNSX, H460a-LNSX, and H460a cells ( $10^3$ /mouse) were injected endotracheally and the mediastinal block was harvested after 4 weeks. Mice were assessed for tumor growth without knowledge of the treatment group. Seven of 9 mice inoculated with H460a-LNSX (mean volume  $12.5 \pm 2.2$  SE  $\text{mm}^3$ ) and 12 of 14 mice inoculated with H460a parental cells (mean volume  $39.9 \pm 4.25$  SE  $\text{mm}^3$ ) had tumors. Only 3 of 17 mice receiving H460a-AS-LNSX cells had tumors (mean volume  $2.95 \pm 1.25$   $\text{mm}^3$ ). From these studies, it is concluded that 1) retroviral gene transduction can be used to express anti-sense constructs in human tumor cells at levels that mediate a biologic effect; 2) AS-mediated inhibition of activated K-ras expression effectively inhibits proliferation and tumorigenicity of human cancer cells. Expression of the AS-LNSX expression in the H460a cells has been stable up to 6 months.

#### B. Restoration of expression of wtp53 gene product

##### 1. Preliminary studies with plasmid DNA

The p53 gene is the most commonly altered gene yet described in human cancers. To study this gene, a cell culture model system of cell lines varying in p53 expression was established. The H322a lung adenocarcinoma cell line expresses the mutant p53 protein as shown by the presence of high levels of endogenous p53 mRNA and phosphorylated protein. We showed that the H322a cell line has a G:T transversion at codon 248 (Arg to Leu) with absence of the wildtype allele. The H358a cell line has a homozygous p53 deletion. The H460a and H226b cell lines are homozygous for the wildtype p53. Expression vectors for sense (S-p53) and antisense p53 (AS-p53) cDNA with a  $\beta$ -actin promoter were constructed to study the effect of wtp53 expressed in lung cancer cells with mutant or deleted p53 and the effects of reducing wildtype and mutant p53 expression. (Mukhopadhyay et al., 1991)

Stable transfectants of p53 mutant cells (H322a) or deleted p53 (H358) expressing S-p53 could not be rescued. Failure to isolate colonies expressing sense p53 RNA in cells with homozygous mutant or deleted alleles shows that

wtp53 can suppress transformation in cancer cells expressing a mutant p53 or having a homozygous p53 deletion.

In general, transfection with AS-p53 reduced colony formation (10-fold) by cells with endogenous mutant p53. This indicates that expression of mutant p53 contributes to the transformed phenotype. As expected, cells with wtp53 (H226b) showed increased tumorigenicity when transfected with AS-p53. The H226b cells expressing AS-p53 grow significantly more rapidly in nu/nu mice than the cells transfected with the control plasmid. This indicates that elimination of the wtp53 gene product enhances features of the malignant phenotype.

The inventors studies showed that wtp53 is dominant and can suppress the malignant phenotype in cells with mutant or deleted p53. The presence of the mutant p53 confers transforming potential to the gene product, which can be suppressed by AS-p53. Thus, in cancer cells both the absence of wtp53 and the presence of certain p53 mutations may enhance the malignant phenotype.

#### 2. Gene construct

The retroviral vector construct contains p53 cDNA with its  $\beta$ -actin promoter inserted into the LNSX vector (Miller et al., 1989; Palmer et al., 1987) in a reverse orientation, in essentially the same manner as described for the p21 AS embodiments.

#### 3. Packaging

See section A.2. above

#### 4. Preclinical studies

The LNSX-p53 and the DC-p53 were transduced into H322a (mutant p53), H358a (deleted p53), and H460a (wt p53). H322a cells that underwent one cycle of infection with the wtp53 construct but without G418 selection had an over 3-fold reduction in proliferation compared to cells that received either the unmodified vector or no treatment. Two cycles of transduction without G418 selection resulted in a 5-fold reduction in proliferation (FIG. 13). A similar result was observed for the H358a cell line when transduced with LNSX-p53. The proliferation of the H460a cell line which has a wildtype p53 was not altered by transduction with any of the p53 retroviral constructs (FIG. 14). Thus, retroviral mediated gene transfer of wtp53 into human lung cancer cells with deleted or mutated p53 significantly reduces the proliferation of those cells. The expression of the mutated p53 protein is uniform in cultured cell lines as detected by immunohistochemistry. In fresh lung tumors that express high levels of p53 protein, expression is detected in >90% of cells.

A critical question is the ability of the retroviral constructs to transduce established tumor cells in vivo. This question was addressed by injecting H460a ( $10^5$ ) cells in the mouse right mainstem bronchus followed 3 days later by lavage with LNSX retroviral supernatant ( $10^6$  CFU in 0.1 ml). LNSX was used so that the neo gene could be used as a marker for transduction. It was necessary to recover tumor cells for analysis so that the AS construct was not used. Tumors were harvested and the presence of the neo gene was assessed by Southern hybridization. The neo gene was detected in the DNA from the H460a cells indicating successful transduction of the retrovirus 30 days after lavage. Although this data is encouraging, the model has limitations. Direct injection of endobronchial tumor is not possible in this model. Other sites of direct injection do not accurately simulate the milieu of endobronchial lung cancer. Thus, definitive answers concerning efficacy must be obtained through this clinical trial.

#### C. Treatment Plans

In proposed preferred treatment protocols, patients will undergo bronchoscopy to assess the degree of obstruction.

As much gross tumor as possible should be resected endoscopically. Patients should preferably undergo bronchoscopy under topical or general anesthesia. A Stifcor™ transbronchial aspiration needle (21 g) will be passed through the biopsy channel of the bronchoscope. The residual tumor site will be injected with  $10^7$  CFU of the appropriate retroviral supernatant. The volume will be no greater than 10 ml. Protamine will be added at a concentration of 5  $\mu$ g/ml. This is 0.2% of the amount given intravenously to reverse heparinization.

Injections will be circumferential and will be intratumor and submucosal. The AS-K-ras supernatant will be used for K-ras mutations and the p53 supernatant will be used for p53 mutations. The injections will be repeated daily for five consecutive days. The treatment will be repeated monthly.

#### 1. Criteria for response and toxicity

There are various criteria that one should consider as presenting the existence of a need for response or the existence of toxicity. To assist in determining the existence of toxicity, the tumor bed should be photographed prior to a course of therapy. The longest diameter and its perpendicular will be measured. Size will be reported as the product of the diameters. From these data, one can calculate from these numbers the rate of regrowth of the tumor.

The time to progression can also be measured from the first observation with reduction in tumor bulk until there is evidence of progressive disease. Progressive Disease is defined as an increase of  $\geq 25\%$  in the sum of the products of the diameters of the measured lesion. Patients must have received at least two courses of therapy before a designation of progression is made. The survival of patients will be measured from entry into protocol.

#### 2. Potential risks of retroviral gene transduction

The possibility of causing malignancy in normal cells secondary to random insertion of the retroviral vector in the genome exists although this risk is thought to be very low. Tests of viral supernatant will be conducted to assure that no replication competent virus is present. Non-replicating bronchial epithelial cells will not take up the vector.

#### 3. Risk from murine retrovirus

The retrovirus derived from the Moloney murine leukemia virus is modified so that it no longer contains intact viral genes. Thus, it cannot produce an intact infectious virus. Assays may be performed on the retroviral vector supernatant and the packaging cell to insure that replication competent virus is not present. Extensive safety studies have been performed on this retroviral construct in primates. Large infusions of infectious murine amphotrophic virus produce no acute pathologic effects. Primates have also received retroviral gene-modified autologous bone marrow cells with no evidence of toxicity as long as 4 years after infusion.

#### 4. Efficacy of aminoglycoside antibiotics

The neomycin resistance gene product, neomycin phosphotransferase, phosphorylates the 3' hydroxyl group of the aminohexose I of neomycin and its analogues. Amikacin, but not gentamicin and tobramycin which do not contain an hydroxyl at the 3" position, is inactivated. Thus, induction of the neomycin resistance gene would not exclude aminoglycosides or any other conventional antibiotic from use in these patients.

#### 5. Criteria for discontinuing therapy

There are various criteria that one should consider employing in making a decision to discontinue therapy. For example, an increase in the endobronchial tumor after a minimum of 2 or more courses of therapy, or the development of unacceptable toxicity defined as unpredictable,

irreversible, or Grade 4. Patient refusal of therapy due to a specific toxicity should be graded as 4 and an explanatory note recorded. One should also consider discontinuing therapy upon the occurrence of significant hemoptysis, coagulopathy, or progressive postobstructive pneumonia.

The present invention has been disclosed in terms of preferred modes found to work well in the practice of the invention. However, numerous modifications and changes in the steps, procedures used and material will become apparent to those of skill in the art in light of the disclosure. All such modifications are intended to be within the spirit of the present invention and scope of the appended claims.

#### REFERENCES

The following references are hereby incorporated by reference to the extent that they describe, explain, teach methodology for or provide useful materials or compositions for use in connection with the practice of the present invention, for the reasons specified in the foregoing text.

Barbacid, et al. (1987), *Genes. Ann. Rev. Biochem.*, 56:779.

Becker-Andre, et al. (1989), *Nucleic Acids Res.*, 17 (22):947.

Bishop J M. Molecular themes in oncogenesis. *Cell* 1991; 64:235-248.

Bishop (1987) *Science*, 235:305-311.

Bodine, D. M., McDonagh, K. T., Seidel, N. E. & Nienhuis, A. W. (1990). *Ann NY Acad Science* 612, 415-427.

Bos (1989), *Cancer Res.*, 49:4682-4689.

Bos, et al. (1987), *Nature*, 327:293-297.

Bos, J. L., Toksoz, D., Marshall, C. J., Verlaan-de Vries, M., Veeneman, G. H., Van Der Eb, A., Van Boom, J. H., Janssen, J. W. G. & Steenvoorden, A. C. M. (1985). *Nature* 315, 726-730.

Bressac B, Galvin K M, Liang T J, Isselbacher K J, Wands J R. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; 87:1973-1977.

Brown C C, Kessler L G. Projections of lung cancer mortality in the United States: 1985-2025. *JNCI* 1988; 80:43-51.

Brown, et al. (1989), *Oncogene Research*, 4:243-252.

Buvoli, et al. (1983), *Nature*:304:497-500.

Casson A G, Mukhopadhyay T, Cleary K R, Ro J Y, Levin B, Roth J A. p53 gene mutations in Barrett's epithelium and esophageal cancer. *Cancer Res* 1991; 51:4495-4499.

Chang, E. H., Miller, P. S., Cushman, C., Devadas, K., Dirollo, K. F., Tso, P. O. P. & Yu, Z. P. (1991). *Biochem* 30, 8283-8286.

Chen P-L, Chen Y, Bookstein R, Lee W-H. Genetic mechanisms of tumor suppression by the human p53 gene. *Science* 1990; 250:1576-1580.

Chomczynsky, et al. (1987), *Anal. Biochem.*, 162:156-159.

Cline, et al. (1987), *Cancer*, 60:2669-2674.

Cooper (1982), *Science*, 218:801-806.

Debus N, Berdichevsky F B, Gryasnov S M. Effects of antisense oligodeoxynucleotides complementary mRNA of the human c-Harvey-ras oncogene on cell proliferation. *J Cancer Res Clin Oncol* 1990; 116 (Suppl., Part 1):S-162.(Abstr)

Delauney, et al. (1988), *Proc. Natl. Acad. Sci. USA*, 85:4300-4304.

Der, et al. (1982), *Proc. Natl. Acad. Sci. USA*, 79:3637-3640.

Dolcetti R, Maestro R, Feriotto G, Pelucchi S, Rizzo S, Boiocchi M. p53 genetic abnormalities in human squamous cell carcinomas of the larynx. *Oncogene* 1990; 6:44-45.

Ellis, et al., (1981), *Nature*, 292:506-511.

Emerman, M. & Temin, H. M. (1986). *Mol Cell Biol* 6 (3), 792-800.

Emerman, M. & Temin, H. M. (1984). *Cell* 39, 459-467.

Feig, et al. (1984), *Science*, 223:698-701.

Fidler, I. (1986). *Cancer Metast Rev* 5, 24-49.

Graham, F. L. & Van Der Eb, A. J. (1973). *Virology* 52, 456-467.

Gunning, P., Leavitt, J., Muscat, G., Ng, S-Y. & Kedes, L. (1987). *Proc Natl Acad Sci USA* 84, 4831-4835.

Hamtzopoulos, et al. (1989), *Proc. Natl. Acad. Sci. U.S.A.*, 86:3519.

Hollstein M, Sidransky D, Vogelstein B, Harris C C. p53 mutations in human cancers. *Science* 1991; 253:49-53.

Howard, R. B., Chu, H., Zeligman, B. E., Marcell, T., Bunn, P. A., McLemore, T. L., Mulvin, D. W., Cowen, M. E. & Johnston, M. R. (1991). *Cancer Res* 51, 3274-3280.

Humphrey E W, Smart C R, Winchester D P, et al. National survey of the pattern of care for carcinoma of the lung. *J Thorac Cardiovasc Surg* 1990; 100 (6):837-843.

Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D. & Gilman, A. G. (1984). *Science* 226, 860-862.

Kasid, et al. (1989), *Science*, 243:1354-1356.

Khokha, et al. (1989), *Science*, 243:957-960.

Komaki, R., Garden, A. S. and Cundiff, J. H. Endobronchial Radiotherapy. In: *Advances in Lung Cancer*, edited by Roth, J. A., Hong, W. K. and Cox, J. D. Cambridge, Mass.: Blackwell Scientific Publications, Inc., 1992.

Krontiris, et al. (1981), *Proc. Natl. Acad. Sci. USA*, 78:1181-1184.

Kumar, et al. (1990), *Science*, 248:1101-1104.

Lane, et al. (1982), *Cell*, 28:873-880.

Lane D P, Benchimol S. p53: oncogene or anti-oncogene? *Genes & Develop* 1990; 4:1-8.

Lee et al., EP Publication No. 0 475 623 A1, 1992

Mabry, et al. (1988), *Proc. Natl. Acad. Sci. USA*, 85:6523-6527.

Mann R, Mulligan R C, Baltimore D. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 1983; 33:153-159.

Markowitz D, Goff S, Bank A. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* 1988; 167:400-406.

Miller, D. G., Adam, M. A. & Miller, A. D. (1990). *Mol Cell Biol* 10 (8), 4239-4242.

Miller, A. D. & Rosman, G. J. (1989). *BioTechniques* 7 (9), 980-990.

Miller A D, Rosman G J. Improved retroviral vectors for gene transfer and expression. *BioTechniques* 1989; 7(9):980-990.

Mitsudomi, T., Steinberg, S. M., Oie, H. K., Mulshine, J. L., Phelps, R., Viallet, J., Pass, H., Minna, J. L. & Gazdar, A. F. (1991) *Cancer Res* 51, 4999-5002.

Mukhopadhyay T, Tainsky M, Cavender A C, Roth J A. Specific inhibition of K-ras expression and tumorigenicity of lung cancer cells by antisense RNA. *Cancer Res* 1991; 51:1744-1748.

Mukhopadhyay T, Cavender A, Tainsky M, Roth J A. Expression of antisense K-ras message in a human lung cancer cell line with a spontaneous activated K-ras oncogene alters the transformed phenotype. *Proc Amer Assoc Cancer Res* 1990; 31:304.

Mukhopadhyay T, Cavender A C, Branch C D, Roth J A. Expression and regulation of wild type p53 gene (wtp53) in human non-small cell lung cancer (NSCLC) cell lines carrying normal or mutated p53 gene. *J Cell Biochem* 1991; Suppl 15F:22.

Murray, et al. (1981), *Cell*, 25:355-361.

- Neckers, L., Whitesell, L., Rosolen, A. & Geselowitz, D. A. (1992). *Crit Rev Oncogenesis* 3, 175-231.
- Nigro J M, Baker S J, Preisinger A C, et al. Mutations in the p53 gene occur in diverse human tumor types. *Nature* 1989; 342:705-708.
- Owens, G. C. & Boyd, C. J. (1991). *Development* 112, 639-649.
- Paget, S. (1989). *Lancet* 1, 571-573.
- Palmer T D, Hock R A, Osborne W R A, Miller A D. Efficient retrovirus-mediated transfer and expression of a human adenosine deaminase gene in diplid skin fibroblasts from an adenosine deaminase-deficient human. *Proc Natl Acad Sci USA* 1987; 84:1055-1059.
- Papageorge, et al. (1982), *J. Virol.*, 44:509-519.
- Parada, et al. (1982), *Nature*, 297:474-478.
- Perez C A, Stanley K, Rubin P, et al. Patterns of tumor recurrence after definitive irradiation for inoperable non-oat cell carcinoma of the lung. *Int J Radiat Oncol Biol Phys* 1980; 6:987-994.
- Perucho, et al. (1981), *Cell*, 27:467-476.
- Prochownik, et al. (1988), *Mol. Cell. Biol.*, 8:3683-3695.
- Pulciani, et al. (1982), *Nature*, 300:539-542.
- Pulciani et al. (1982), *Proc. Natl. Acad. Sci. USA*, 79:2845-2849.
- Reynolds S H, Anna C K, Brown K C, et al. Activated protooncogenes in human lung tumors from smokers. *Proc Natl Acad Sci USA* 1991; 88:1085-1089.
- Rodenhuis S, Slebos R J C, Boot A J M, et al. Incidence and possible clinical significance of K-ras oncogene activation in adenocarcinoma of the human lung. *Cancer Res* 1988; 48:5738-5741.
- Rodenhuis S, Van De Wetering M L, Mooi W J, Evers S G, Van Zandwijk N, Bos J L. Mutational activation of the K-ras oncogene. *N Engl J Med* 1987; 317:929-935.
- Rodenhuis, S. & Slebos, R. J. C. (1990). *Am Rev Respir Dis* 142, 27-30.
- Rodenhuis, et al. (1990), *Proc. Amer. Soc. Clin. Oncol.*, 9:228.
- Rodrigues N R, Rowan A, Smith M E F, et al. p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990; 87:7555-7559.
- Saiki, et al. (1985), *Science*, 230:1350-1354.
- Saison-Behmoaras, T., Tocque, B., Rey, I., Chassignol, M., Thuong, N. T. & Helene, C. (1991). *EMBO J* 10 (5), 1111-1118.
- Santos E, Nebreda A R. Structural and functional properties of ras proteins. *FASEB* 1989; 3:2151-2163.
- Santos, et al. (1984), *Science*, 223:661-664.
- Santos, et al. (1982), *Nature*, 298:343-347.
- Shimizu K, Birnbaum D, Ruly M A, et al. Structure of the ki-ras gene of the human lung carcinoma cell line calu-1. *Nature* 1983; 304:497-500.
- Shimizu, et al. (1983), *Proc. Natl. Acad. Sci. USA*, 80:383-387.
- Shimizu, et al. (1983), *Proc. Natl. Acad. Sci. USA*, 80:2112-2116.
- Sistonen, L. & Alitalo, K. (1986). *Ann Clin Res* 18, 297-303.
- Smith, et al. (1986), *Proc. Natl. Acad. Sci. USA*, 83:2787-2791.
- Soriano P, Montgomery C, Geske R, Bradley A. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* 1991; 64:693-702.
- Spandidos, et al. (1989), *J. Pathol.*, 157:1-10.
- Tabin, et al. (1982), *Nature*, 300:143-149.
- Takahashi et al. (1990), *J. Clin. Invest.*, 86:363-369.
- Takahashi et al. (1992), *Cancer Research*, 52:2340-2343.
- Takahashi T, Nau M M, Chiba I, et al. p53: a frequent target for genetic abnormalities in lung cancer. *Science* 1989; 246:491-494.

- Taparowski, et al. (1982), *Nature*, 300:762-765.
- Taya, et al. (1984), *EMBO J.*, 3:2943-2946.
- Travali, et al. (1990), *FASEB*, 4:3209-3214.
- Vogelstein, et al. (1988), *N. Engl. J. Med.*, 319:525-532.
- Wickstrom, et al. (1988), *Proc. Natl. Acad. Sci. USA*, 85:1028-1032.
- Yamamoto, et al. (1985), *Prog. Med. Virol.*, 32:101-114.
- Yuasa, et al. (1983), *Nature*, 303:775-779.
- Zakut-Houri et al. (1985), *EMBO T.*, 4:1251-1255.
- What is claimed is:
1. A retroviral expression vector comprising a gene expression unit which comprises a wild-type p53 gene under the control of a  $\beta$ -actin promoter, the gene expression unit being positioned to effect transcription of the gene in an orientation opposite that of retroviral transcription.
  2. The vector of claim 1, further defined as a Moloney murine leukemia virus vector.
  3. The vector of claim 1, further comprising a second gene expression unit which includes a selectable marker gene, expressed from a retroviral long-term repeat.
  4. The vector of claim 3, wherein the selectable marker gene comprises a neo gene.
  5. The vector of claim 1, wherein said vector is replication defective.
  6. The vector of claim 1, wherein said vector further comprises a polyadenylation signal.
  7. A pharmaceutical composition comprising the vector of any one of claims 1-6 in a pharmacologically acceptable state.
  8. A method for the preparation of a retroviral expression vector comprising constructing a gene expression unit which comprises a wild-type p53 gene placed under the control of a  $\beta$ -actin promoter, and positioning the gene expression unit into a retroviral vector in an orientation opposite that of retroviral transcription.
  9. A method for treating cancer in a human patient comprising directly introducing into a p53-deficient tumor cell of the patient a retroviral expression vector dispersed in a pharmaceutical diluent, wherein said expression vector comprises a gene expression unit which comprises a wild-type p53 gene under the control of a  $\beta$ -actin promoter, the gene expression unit being positioned to effect transcription of the gene in an orientation opposite that of retroviral transcription, and wherein expression of p53 by said expression vector is effective to inhibit the growth of said tumor cell.
  10. The method of claim 9, wherein the human patient has an epithelial cancer.
  11. The method of claim 9, wherein the human patient has lung cancer.
  12. The method of claim 11, wherein the patient has non-small cell lung cancer.
  13. The method of claim 12, wherein the non-small cell lung cancer is squamous cell cancer.
  14. The method of claim 12, wherein the non-small cell lung cancer is adenocarcinoma.
  15. The method of claim 12, wherein the non-small cell lung cancer is large-cell undifferentiated.
  16. The method of claim 11, wherein the lung cancer is small cell lung cancer.
  17. The method of claim 9, wherein said introducing is via intratumoral injection.
  18. The method of claim 9, wherein said introducing is via circumferential injection of said tumor.
  19. The method of claim 9, further comprising tumor resection.
  20. The method of claim 19, wherein said resection is via bronchoscopy.

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21. The method of claim 9, wherein said introducing is via injection of a resected tumor site.

22. The method of claim 17, wherein said injection is submucosal.

23. The method of claim 17, wherein said injection is subcutaneous.

24. The method of claim 9, wherein said introducing is performed multiple times.

25. The method of claim 24, wherein said introducing is performed daily for five consecutive days.

26. The method of claim 24, wherein said introducing is performed monthly.

27. The method of claim 9, further comprising photographing said tumor mass prior to introducing said retroviral composition.

28. The method of claim 9, wherein said retroviral composition is delivered in 10 ml.

29. The method of claim 9, wherein said retroviral composition is delivered in 0.1 ml.

30. The method of claim 9, wherein said retroviral composition has a titer of at least  $10^5$  CFU/ml.

31. The method of claim 30, wherein said retroviral composition has a titer of at least  $10^6$  CFU/ml.

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32. The method of claim 31, wherein said retroviral composition has a titer of at least  $6 \times 10^6$  CFU/ml.

33. The method of claim 32, wherein said retroviral composition has a titer of at least  $9 \times 10^6$  CFU/ml.

34. The method of claim 9, wherein said retroviral vector is a Moloney murine leukemia virus vector.

35. The method of claim 9, wherein said tumor mass is endobronchial.

36. The method of claim 9, wherein said retroviral composition further comprises protamine.

37. The method of claim 36, wherein said protamine is present at a concentration of  $5 \mu\text{g/ml}$ .

38. The method of claim 9, wherein said expression vector further comprises a second gene expression unit which includes a selectable marker gene, expressed from a retroviral long-term repeat.

39. The method of claim 38, wherein the selectable marker gene comprises a neo gene.

40. The method of claim 9, wherein said expression vector is replication defective.

41. The method of claim 9, wherein said expression vector further comprises a polyadenylation signal.

\* \* \* \* \*



# Expert Opinion on Investigational Drugs

Volume 4 Number 6 June 1995

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## Section Review

### Oncologic, Endocrine & Metabolic

#### Gene therapy strategies for cancer

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Gene therapy of cancer has undergone an explosive development at its infant stage during the past five years. It is an attractive biotechnology not because it has surpassed conventional cancer therapies but for its potential to provide scientists and clinicians with powerful tools to cure cancer through genetic manipulations. Due to the application of molecular biology techniques to cancer treatment, through intervention in the mechanisms of carcinogenesis, the field of cancer gene therapy is filled with opportunities for innovative design of therapeutic strategies. Based on an overview of the field, this article organises current technologies in cancer gene therapy into six major approaches:

- 1) Genetic sequence-targeted therapies;
- 2) Tumour suppressor gene therapy;
- 3) Toxin or prodrug-activation gene therapy;
- 4) Drug-resistance gene therapy;
- 5) Cytokine gene therapy and tumour vaccination;
- 6) Combinational gene therapy.

On learning about these technologies, each of which may contain several distinct methodologies, it is easy to see that the field of cancer gene therapy is currently in a state of dynamic development.

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#### Introduction

Cancer is a disease of genes. There is ample evidence that carcinogenesis is a multistage process involving multiple genetic and epigenetic events in proto-oncogenes, tumour suppressor genes, and antimetastasis genes [1,2]. Chemicals, radiation, and viruses all can initiate cellular transformation by attacking genetic material, for example, activating proto-oncogenes or inactivating tumour suppressor genes. The initiated cells, promoted by epigenetic factors and further genetic alterations, can expand themselves and their

defects: dysregulated terminal differentiation, lost control of growth, and acquired resistance to cytotoxic effects. This expansion leads to preneoplastic lesions, which progress further through the process of epigenetic influence and genetic disorder and finally reach the stage of clinical cancer.

Gene therapy of cancer is, therefore, a rational strategy for cancer treatment. The potential effectiveness of gene therapy is promised not only by its precise targeting at the mechanisms of the disease, but also by its genetic approaches which are based on rapidly advancing molecular biotechnology.

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- Tumour suppressor gene therapy

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- Toxin gene therapy
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- Multidrug resistance gene and other chemoresistant genes
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### Cytokine gene therapy and tumour vaccination

- Genetic modulation of lymphocytes
- Cytokine gene-modified tumour cell vaccination
- Antigen presentation gene-modulation for tumour cell vaccination

### Combinational gene therapy

### Prospects

Of all of the gene therapy clinical protocols thus far approved by the federal regulatory agencies, more than 85% are cancer gene therapy trials, indicating that gene therapy of cancer currently has a particular favour for research, development, and application.

There are several reasons for the explosive development of cancer gene therapy from experimental hypotheses to clinical trials:

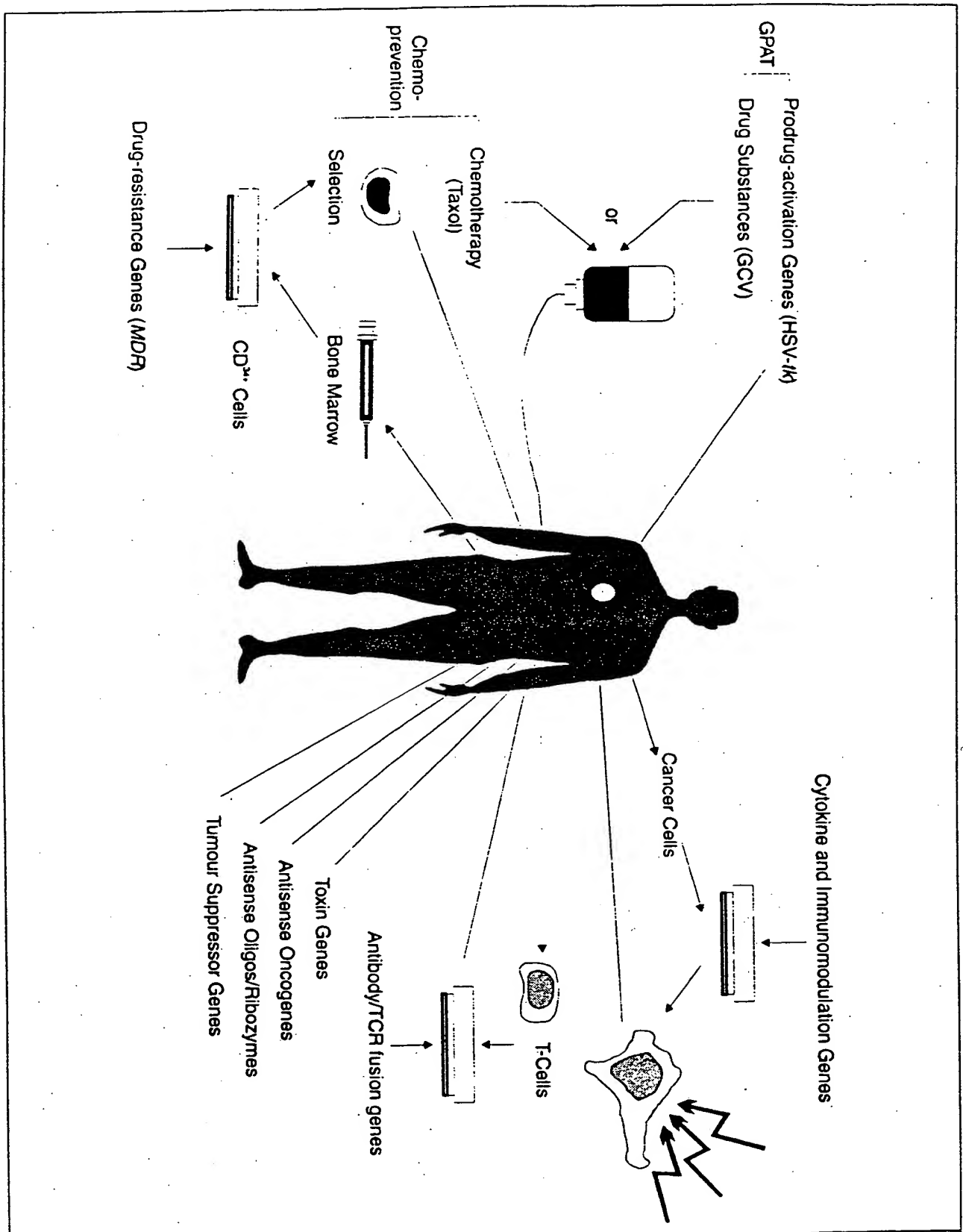
- the discovery of the genetic basis of cancer inspired the development of genetic approaches to cope with the disease;
- the availability of gene or antigene manipulation technology which was promoted by successful gene therapy of somatic or metabolic genetic diseases;
- the unsatisfactory status of conventional cancer therapies; and

## Abbreviations

Ad: adenovirus  
 AML: acute myeloblastic leukaemia  
 APC: antigen-presenting cell  
 APC: adenomatosis polyposis coli tumour suppressor gene  
 araATP: adenine arabinonucleoside triphosphate  
 araM: 6-methoxypurine arabinonucleoside  
 BRCA1: breast and ovarian cancer susceptibility gene  
 cdk: cyclin-dependent kinase  
 CFTR: cystic fibrosis transmembrane conductance regulator  
 Cip1: cyclin-dependent kinase-interacting protein 1  
 CML: chronic myelogenous leukaemia  
 CTL: cytotoxic T-lymphocyte(s)  
 DCC: deleted in colon cancer tumour suppressor gene  
 G-CSF: granulocyte colony-stimulating factor  
 GCV: ganciclovir  
 GM-CSF: granulocyte-macrophage colony-stimulating factor  
 GPAT: genetic prodrug-activation therapy  
 GSH: L-g-glutamyl-L-cysteinglycine  
 GST: glutathione-S-transferase  
 HPV: human papilloma virus  
 HSV-*tk*: herpes simplex virus-thymidine kinase gene  
 IFN: interferon  
 IL: interleukin  
 LAK: lymphokine-activated killer cell  
 LLC: Lewis lung carcinoma  
 MDR: multidrug resistance  
 MDR: multidrug resistance gene  
 MHC: major histocompatibility complex  
 MnSOD: manganese superoxide  
 MTS: major tumour suppressor  
 NDP: nucleotide diphosphate  
 Neo<sup>R</sup>: neomycin-resistant gene  
 NF1: neurofibromatosis tumour suppressor gene  
 PBL: peripheral blood lymphocyte(s)  
 PFU: plaque-forming unit  
 PKC: protein kinase C  
 Rb: retinoblastoma susceptibility gene  
 TCR: T-cell receptor  
 TIL: tumour-infiltrating lymphocytes  
 TNF- $\alpha$ : tumour necrosis factor- $\alpha$   
 Topo II: topoisomerase II  
 VDEPT: virus-directed enzyme/prodrug therapy  
 VZV-*tk*: varicella-zoster virus thymidine kinase gene  
 WAF1: wild-type p53-activated fragment 1  
 WTI: Wilms tumour suppressor gene

- the desperation of patients who are willing to try novel therapies in the face of a life-threatening disease.

Diverse strategies and innovative approaches to cancer gene therapy have been developed. Figure 1 depicts the current major methodologies of cancer gene therapy. Despite the great potential and rapid advances in this technology, the development of cancer gene therapy remains at a very early stage. The objective of this review is to present an overview of the current state of the field, which may be used as a reference for understanding the prospects of cancer gene therapy.



**Figure 1: Major approaches of gene therapy for cancer.** Shown is a diagrammatic summary of the strategies of cancer gene therapy. A hypothetical tumour located in the lung is depicted either as a source for cancer cell isolation or as a target for gene transfer. Each approach is discussed in detail in the text. GPAT, genetic prodrug-activation therapy; HSV-*tk*: herpes simplex virus-thymidine kinase gene; GCV: ganciclovir; *MDR*: multidrug resistance gene; Taxol: paclitaxel; TCR: T-cell receptor; Oligos: oligodeoxynucleotides.

### Genetic sequence-targeted therapies

The past five years have witnessed a rapid development of genetic sequence-targeted therapeutic agents. The significant advances in research of gene regulation of cells and molecular mechanisms of diseases have given pharmaceutical scientists a rational basis for the design of drugs that target genetic sequences through specific recognition of and by hydrogen bonding between complementary bases.

Along the pathway for genetic information flow from DNA to protein, several interventions have been developed to modulate gene expression and regulation. Examples include:

- triplex formation of oligonucleotides with double-stranded DNA to block transcription;
- antisense oligonucleotides;
- antisense RNA binding single-stranded DNA or mRNA to interfere with transcription, splicing, and translation;
- ribozymes specifically binding and cleaving target mRNA.

All of these approaches have been demonstrated to be effective in cellular treatments or even in animal models. Their applications for cancer gene therapy are under investigation. Besides these, new approaches are under development, such as oligonucleotides covalently linked to an intercalating agent or to a nucleic acid-cleaving reagent [3], peptide nucleic acids, which possess both antisense and antigene properties [4], and sense oligonucleotide to block transcription factors competitively, or site-specific DNA-binding proteins to block the transcription of oncogenes [5]. On antisense approaches to cancer gene therapy, a recent review published in *Cancer Gene Therapy* provides additional references for and future directions of this type of technology [6].

#### Antisense oligonucleotides

The first antisense oligonucleotide to be administered to humans, ISIS 2105, is in Phase II clinical tests [7]. The rapid progress in the development of antisense oligonucleotide drugs has been attributed to the synthetic oligonucleotide technology, which allowed creation of a variety of modified oligonucleotides to cope with the rapid degradation of regular oligonucleotides by nucleases *in vivo* [8]. The most promising of the modified ones are phosphorothioate oligonucleotides, which have been shown to be effective against cellular RNA and have attractive pharmacokinetic and toxicological properties in animals [9,10].

Anti-oncogene oligonucleotides have been demonstrated in a variety of cancer cell lines to inhibit effectively the activities of different oncogenes or proto-oncogenes, such as *c-abl*, *c-fos*, *c-fes*, *c-fms*, *c-kit*, *c-myc*, *c-myc*, *c-raf*, *c-src*, and *ras* [9,11,12]. Specific inhibition of p210 mRNA of the *bcr-abl* fusion gene in chronic myelogenous leukaemia (CML) by antisense oligonucleotides is an often cited example [13,14]. Inhibition of gene expression and tumourigenicity by antisense oligonucleotides has also been shown in several tumour or other animal models [15].

In 1992, an antisense oligonucleotide designed to block expression of the *p53* gene was administered systemically to a patient with acute myeloblastic leukaemia (AML) [16]. This was based on the previous observation that *p53*, though being currently considered as a tumour suppressor, was correlated with the proliferation of AML blast stem cells [17]. No major toxicity was detected from this patient after given a ten-day infusion of the anti-*p53* oligonucleotide at a dose of 0.05 mg/kg/hour (total dose: 700 mg). This led to a Phase I dose escalation study with more patients. The 20-mer anti-*p53* (exon 10) oligonucleotides had a strong inhibitory effect on the *in vitro* growth and viability of leukaemic blasts from more than thirty patients with AML as compared with medium alone, or with two oligonucleotide controls.

The mechanism by which antisense oligonucleotides inhibit gene expression has not been clearly demonstrated by *in vivo* evidence. The initial thought was that they bound to target mRNA and induced translation arrest [18]. This mechanism may be of importance when the antisense oligonucleotides are targeted to the translation start codon. The oligonucleotides targeting coding sequences of mRNA may induce RNase H to cleave the DNA-bound RNA, inhibiting translation [19]. Several other mechanisms have been postulated:

- the antisense oligonucleotides interfere with transcription by hybridising to single-stranded DNA;
- they inactivate splicing by binding to hnRNA splicing sites;
- they block RNA transportation from nucleus to cytoplasm; and
- they inhibit the initiation translation by binding to ribosomal subunit entry sites.

Precise molecular targeting mechanisms position antisense oligonucleotides as a type of 'informational drug'. Their efficacy in suppressing gene expression has been well established but does not eliminate the pharmacokinetic limitations conferred by the artificial-oligomer nature of the drugs. Lack of delivery speci-

ficity, instability *in vivo*, preferential accumulation in liver and kidneys, low cellular uptake efficiency, and short retention of effective concentrations within cells are the major problems to be resolved for using antisense oligonucleotides systemically or semi-systemically in the clinic. Although the entry of the oligonucleotide into cells has been shown through receptor-mediated endocytosis [20,21] or recently *via* protein kinase C (PKC)-dependent pinocytosis [22], the internalisation pathways cause the drugs to be retained or destroyed in the cellular vesicle system, limiting their ability to reach action sites at an effective concentration. The receptor-mediated pathway also poses the potential problem that drug uptake will be dependent on the availability of the receptor on the cell surface.

Current approaches to improving the *in vivo* stability and delivery of antisense oligonucleotides range from modification of oligonucleotides to construction of oligonucleotide-liposome complexes [23]. Examples include end modification [24], lipophile conjugation [25], polylysine conjugation with liposome encapsulation [26], and linking of antibodies with oligonucleotide-liposome complex [27]. At one time, researchers wondered whether antisense oligonucleotides were the real 'magic bullet', but the evidence collected thus far does not support this [12]. It is feasible that they could become novel antiviral agents, but much more development is needed before they can confidently be called a new type of anticancer agent. Additional information can be obtained in a recent review on this subject [28].

### Antisense RNA

Antisense RNAs occur naturally in prokaryotic and eukaryotic cells. They have been shown to play regulatory roles in several cellular processes, including DNA replication, transcription, RNA processing, and translation, all through a base-pairing mechanism [29,30]. Their ability to inhibit gene expression specifically led to the use of artificial antisense RNA to study biological function in both prokaryotic and eukaryotic systems [31,32]. This was soon exploited for the development of new approaches for therapy against viruses and cancer.

Antisense RNAs in their DNA template forms can be constructed in expression cassettes, carried by plasmids or viral vectors, and produced either constitutively or following induction in targeted cells. In this aspect, antisense RNAs have an obvious advantage over antisense oligonucleotides, since their templates can be efficiently delivered into target cells by viral vectors or other means and their active forms can be produced within the cells in a controllable manner by different promoters.

Suppression of transcription or translation of proto-oncogenes or oncogenes by antisense RNA has been successfully demonstrated on *c-fos* [33], *c-myc* [34], and *K-ras* [35], in each case leading to reversion of the transformation phenotypes of the target cells. The *K-ras* experiment used a novel design of the antisense RNA template in which a 2 kb *K-ras* genomic fragment containing the first and second exons with the first intron in between was used for specifically attacking *K-ras* mRNA. This strategy was further utilised for generating an anti-*K-ras* recombinant retrovirus [36]. The virus was shown to have a strong inhibitory effect on the growth and tumourigenicity of human lung cancer cells that have a mutated *K-ras*.

It was also shown greatly to reduce tumour formation in mouse models of orthotopic human lung cancer [37]. These findings led to the development of a clinical trial protocol that has been approved by the NIH for the treatment of unresectable lung cancer by direct intratumour injection of the anti-*K-ras* recombinant retrovirus.

A set of mechanisms for the activity of antisense RNA have been proposed that are very similar to those for antisense oligonucleotides:

- the antisense RNA interferes with transcription;
- it blocks RNA splicing and exportation;
- it inhibits translation; and
- it induces RNase III to cleave double-stranded RNA after binding to target mRNA.

*In vivo* evidence of these mechanisms has not been found. Double-stranded RNA complexes have rarely been isolated. There are unique problems associated with the antisense RNA approach, such as the formation of self-inhibitory secondary structure, the degradation by nuclease, and the variations in expression level and persistence. Further development of this approach will focus on optimising regulation of antisense RNA expression and producing targeted, efficient delivery for DNA constructs.

### Antigene oligonucleotides

The antigen oligonucleotide approach extends from the strategy of antisense oligonucleotides. Antigene oligonucleotides bind to double-stranded DNA through Hoogsteen hydrogen bonding [38], which, distinct from Watson-Crick base-pairing, occurs between polypurine or polypyrimidine oligodeoxynucleotides and double-stranded polypurine or polypyrimidine stretches in DNA. Antigene oligonucleotides occupy the major groove of the DNA helix and specifically suppress the activities of targeted genes. The biological effects of antigen oligonu-

cleotides were first shown by their blocking the access of sequence-specific proteins to the same or neighbouring sequences [39,40]. Homopyrimidine oligonucleotides bound to the homopurine-homopyrimidine sequence inhibited restriction enzyme cleavage and/or methylation by methylases. Binding of a transcription factor, Sp1, was inhibited by a triplex-forming oligonucleotide that overlapped the Sp1 binding site by four base pairs. Specific inhibition of transcription by formation of triplex complexes was demonstrated in the transcription assays of the human *c-myc* gene *in vitro* [41] and the interleukin-2 (IL-2) receptor gene *in vivo* [42].

Two major types of DNA triplexes have been described: intramolecular and intermolecular [43]. In an intermolecular triplex, a separate third strand associates with a target duplex DNA. In an intramolecular triplex, the third strand is a portion of one of the strands of the duplex that has folded back to associate with the purine-pyrimidine tract. The triplex DNA structures have been confirmed by x-ray crystallography, nuclear magnetic resonance, ultraviolet absorption spectroscopy, circular dichroism, enzymatic probing, and chemical probing [43].

The antigene strategy requires that the target sequence be accessible within the chromatin structure in the nucleus. It was shown that the oligonucleotide can reach the nucleus of cells that are incubated with a micromolar concentration of the oligonucleotide [44], but a large part of the oligonucleotide remained trapped in endocytic vesicles and was not available to induce biological effects. Efforts to improve cellular uptake of antigene oligonucleotides have been similar to those used for antisense oligonucleotides. To increase stability, nuclease-resistant oligonucleotides, such as oligo-[ $\alpha$ ]-deoxynucleotides, which can be synthesised with the  $\alpha$ -anomers of nucleotide units, have been applied [45]. Strategies for improving the effectiveness of antigene oligonucleotides include:

- modification of oligonucleotides with unnatural residues [43];
- linkage of oligonucleotides with intercalating or cross-linking agents; and
- conjugation with reactive groups such as cleaving reagents [46].

The antigene oligonucleotide approach is in the early development stage. It has constraints similar to those of antisense oligonucleotides, e.g., instability, lack of delivery specificity, low efficiency of cellular uptake, and inability to sustain effective concentrations in target cells. Besides, its activity is limited to the availability of polypurine stretches in the targeted gene. If

these weaknesses can be overcome, the antigene oligonucleotide strategy is potentially useful for cancer gene therapy.

### Ribozymes

Ribozymes are RNA molecules that possess specific catalytic activities [47]. Among different types of ribozymes [48,49], the best characterised group for the purpose of gene therapy is the hammerhead ribozymes. The consensus hammerhead model consists of three base-paired stems, I, II, and III, and a core region in which thirteen residues are strictly conserved for cleaving immediately 3' to any GUX sequence (where X is C, A, or U).

In the design and construction of a hammerhead ribozyme, specific sequences can be introduced in stems I and III that impose complementary recognition between the ribozyme and target RNA. Hammerhead ribozymes can perform a true enzymatic reaction, during which a substrate is cleaved and the ribozyme itself is not altered, thereby enabling multiple reaction cycles.

The utility of hammerhead ribozymes specifically designed for inhibiting oncogene activities has been demonstrated by anti-*ras* ribozymes [50,51]. In one study, a ribozyme was specifically designed to cleave the H-*ras* mRNA at the mutated codon 12 in human bladder carcinoma EJ cells. The ribozyme, encoded by a synthetic DNA, was carried by a eukaryotic expression vector and transfected into EJ cells. The expressed ribozyme significantly altered the morphology and suppressed the growth of the EJ cells *in vitro*. Reductions in the expression of H-*ras* mRNA and p21 protein in the EJ cells were shown in reciprocal by the increase in expression of the ribozyme RNA. The tumourigenic potential of the EJ cells was greatly inhibited by the ribozyme in a mouse model. Another study used a set of similar ribozymes also targeted at the mutated codon 12 of the H-*ras* mRNA. Plasmids containing the ribozyme-encoding sequences were stably transfected in NIH3T3 cells, which were then transfected with the activated H-*ras* gene. The ribozymes were found to inhibit transformation of NIH3T3 cells by the H-*ras* oncogene. Both of the studies suggested that anti-oncogene ribozymes may be developed as a new class of anticancer agents.

Like other RNA molecules, exogenous ribozymes in target cells suffer from rapid degradation by nucleases, which can directly affect the concentration of the ribozymes and subsequent cleavage efficacy. Attempts have been made to increase the stability of the ribozymes by modifying their primary structure, for example, by adding an extra sequence at the 3' end of

the molecules [52]. Slow turnover of ribozymes, a result of strong binding between the substrate RNA and ribozymes, is another drawback that affects their efficiency as enzymatic drugs, making them behave more like antisense RNA in cells. Targeted delivery of ribozyme constructs to cancer cells *in vivo* remains to be developed. A retroviral vector has been used to carry a ribozyme sequence in the development of an anti-HIV agent [53]. The use of adeno-associated virus vectors to carry ribozyme constructs specific for binding and cleaving the E6 and E7 transcripts of human papillomavirus has been reported [54], and efficient cleavage of the cognate targets *in vitro* were demonstrated under a variety of conditions, including at physiological temperature. Further research and development of ribozyme-mediated *in vivo* cleavage of target transcripts of oncogenes or HIV may lead to clinical trials of this type of approach.

### Tumour suppressor gene therapy

The genetic bases of cancer include abnormalities in oncogenes and/or tumour suppressor genes. Both types have been the targets of cancer gene therapy. Because the cancer-related defects of tumour suppressor genes are usually mutations or deletions, the strategy in tumour suppressor gene therapy thus far developed has been gene replacement therapy: a wild-type tumour suppressor gene is transferred into cancer cells to restore the normal function of the defective gene. Although this approach appears to be straightforward and logical, it does have several technical hurdles to overcome:

- crucial defective gene(s) must be identified for a given type of cancer in order to reverse the malignant phenotype or induce a tumouricidal effect;
- a highly efficient and targeted delivery system is required;
- the therapeutic gene needs to be delivered into enough target cells to elicit a bystander effect; and
- expression of the therapeutic gene must be controllable and not harmful to normal cells.

Moreover, taking into account the fact that cancer is not a monogenic disease, and often comprises multiple lesions in different oncogenes and tumour suppressor genes, it is reasonable to assume that replacement of a single gene will not always be sufficient to reverse the malignant phenotype. However, the current single-gene replacement therapy has provided encouraging data that support further development of this approach.

### Tumour suppressor genes

The fusion of normal and malignant tumour cells has led to the suppression of tumourigenicity in many different combinations. This phenomenon provided the first evidence that the normal genome might contain 'recessive cancer genes' [55]. Following the identification and cloning of the retinoblastoma susceptibility (*Rb*) and *p53* genes, the field has progressed exponentially. Although tumours develop through multiple changes in several genes, a malignant phenotype has been reversed by the introduction of a single chromosome derived from a normal cell, suggesting that a single suppressor gene may be able to overcome the effects of multiple tumour progression-related cytogenetic changes [56]. The human tumour suppressor genes that have been cloned and characterised include *Rb*, Wilms tumour (*WT1*), and neurofibromatosis (*NF1*), which are involved in paediatric cancers; adenomatous polyposis coli (*APC*) and deleted in colon cancer (*DCC*), which contribute to colorectal cancer; and *p53*, which is found in mutated forms in a wide range of human cancers. More recently, the development of animal models using methods to knock out tumour suppressor genes has demonstrated that disruption of either the *Rb* or *p53* gene yields mice that are prone to cancer formation [57]. Understanding these mechanisms has provided the basis for direct gene replacement therapy, by which abnormal tumour suppressor genes can be corrected.

#### Retinoblastoma susceptibility (*Rb*) gene

The *Rb* gene has been shown to be inactivated in virtually all retinoblastomas as well as in a number of adult tumours, such as small cell lung cancer and cancers of the breast, prostate, and bladder. The *Rb* gene product, in its unphosphorylated form, appears capable of blocking the cell cycle in the G1 phase, thereby maintaining cells in a quiescent state. *Rb* is inactivated by phosphorylation; the cell is allowed to proceed to DNA replication and mitosis [58]. Although genetic events other than *Rb* abnormalities may initiate malignant transformation, loss of the *Rb* protein can remove a residual checkpoint of cell-cycle control, thereby further promoting the growth and/or progress of cancer cells.

Introduction of the normal *Rb* gene into retinoblastoma or osteosarcoma cells with inactivated endogenous *Rb* genes affected cell morphology, growth rate, soft agar colony formation, and tumourigenicity in *nu/nu* mice [59]. A similar activity has been shown in human prostate carcinoma cell lines. These findings suggest that the normal *Rb*-encoded protein may be used clinically for adult neoplasms [60].



**p53 gene**

Named the 'molecule of the year' in 1993 by the journal *Science*, the *p53* tumour suppressor gene has been at the centre stage of cancer research, attracting the attention of biologists and clinicians. Of all the known genes with therapeutic potential in cancer, *p53* is the most extensively studied [61,62]. Mutations to the *p53* gene and allele loss on chromosome 17p, where this gene is located, are among the most frequent alterations yet identified in human malignancies. The *p53* protein is highly conserved through evolution and is expressed in most normal tissues. Wild-type *p53* has been shown to be involved in control of the cell cycle [63,64], transcriptional regulation [65,66], DNA replication [67,68], and induction of apoptosis [69-71]. The wild-type *p53* gene can suppress cell transformation and neoplastic cell growth [72-75]. Overexpression of the *p53* gene product is associated with mutations in *p53* and significantly correlates with a poor prognosis [76,77]. In addition to somatic mutations, it has also been reported that germ-line *p53* mutations were associated with Li-Fraumeni syndrome, a familial cancer disease [78]. In studying the mechanism by which the *p53* protein plays a role in cell-cycle control, a *p53*-regulated 21 kDa protein was identified as cyclin-dependent kinase-interacting protein 1 (Cip1) or wild-type *p53*-activated fragment 1 (WAF1) by two independent groups [79,80]. These studies not only revealed a G1 cyclin-dependent kinase control by *p53* through p21, but also connected the *p53* function to that of Rb through p21 and G1 cyclin-dependent kinases. The mechanisms of action of the *p53* tumour suppressor and prospects for cancer gene therapy by reconstitution of *p53* function were discussed in a recent review [81].

**Other tumour suppressor or cancer susceptibility genes**

The *DCC* and *nm23* genes are also candidates for gene replacement therapy, for colon cancer and metastatic cancers, respectively. The *DCC* gene product has sequence homology to fibronectin-like cell adhesion molecules. The deletion of this gene, which is involved in the transmission of signals generated by cell interactions, is thought to be a relatively early event in the development of colon cancer [82]. The *nm23* gene is considered to be a metastasis suppressor gene and to encode nucleotide diphosphate (NDP) kinase. Some types of tumour cells transfected with this gene lost their metastatic potential when applied in animal models [83,84]. Recently, two major events occurred in the area of identification of new tumour suppressor genes or cancer susceptibility genes. First, two highly related members of the cyclin-dependent kinase (cdk) inhibitor family, termed *p16* (major tumour suppressor 1, *MTS1*) and *p15* (*MTS2*), were isolated from the

chromosomal region 9p21 [85,86]. Second, a strong candidate for the breast and ovarian cancer susceptibility gene *BRCA 1* was identified [87]. While *p16* was shown to be deleted or mutated in a wide range of cancer cell lines, *p15* was shown to be a potential effector of TGF- $\beta$ -induced cell cycle arrest [88]. Despite some challenges to the *p16* gene as a major tumour suppressor [89], further studies demonstrated that alteration or deletion of *p16* and *p15* did occur in different types of primary tumours at a lower frequency than that in tumour cell lines [90-93]. Applications of these new genes in cancer therapy will depend on further research.

**Tumour suppressor gene therapy**

The germ cells of patients with Li-Fraumeni syndrome are heterozygous for wild-type and mutant *p53*, whereas the tumours of these patients are homozygous for mutant *p53*. Mice that have a homozygous deleted *p53* developed a variety of tumours as early as six months of age, whereas, the heterozygous mice that contain one wild-type *p53* allele developed tumours at a reduced frequency and at a slower rate [57]. These observations suggest that the reintroduction of a single copy of the wild-type *p53* gene may be able to reverse malignant phenotypes in tumour cells. Several studies demonstrated that expression of the wild-type *p53* gene suppressed proliferation of human tumour cell lines that lack *p53* or express mutant *p53* *in vitro* [73-75,94]. *In vivo* experiments in nude mice also showed that tumour cells expressing wild-type *p53* are no longer tumourigenic or are less tumourigenic than parental cells.

The research in this area is progressing rapidly and has demonstrated that the restoration of wild-type *p53* function in tumour cells could be a very efficient approach to cancer therapy. The early experiments showed that intratracheal instillation of retrovirus containing wild-type *p53* prevented the growth of established orthotopic human lung cancer in *nu/nu* mice. Further studies, using a multicellular tumour spheroid model, also showed evidence that retroviral vectors were capable of penetrating into three-dimensional structures and that exposure of spheroids to a retroviral vector expressing wild-type *p53* induced programmed cell death in lung cancer cells [95]. These findings suggest that this gene replacement technique may be useful as an adjuvant in eliminating residual cancer cells following surgery and primary radiation of lung cancer. Moreover, this approach is feasible for other tumours such as colon cancer, which is also frequently associated with *p53* mutations. Mutations in *p53* are found in Barrett's epithelium, the premalignant precursor lesion to adenocarcinoma of the oesophagus, and are frequently associated with second primary cancers



of the aerodigestive tract [96,97]. This raises the intriguing possibility that cancer could be prevented by reversing genetic mutations in premalignant lesions with local-regional instillation of viral vectors.

To achieve a more efficient delivery of the *p53* gene into lung cancer cells *in vivo*, a replication-defective and helper-independent recombinant *p53* adenovirus was generated [98]. The virus, Ad5CMV-*p53*, carries an expression cassette that contains human cytomegalovirus E1 promoter, human wild-type *p53* cDNA, and SV40 early polyadenylation signal. Human non-small cell lung cancer cell lines representing different *p53* configurations were used to evaluate the expression of the Ad5CMV-*p53* virus. In the H358 cell line, which has a homozygous deletion of *p53*, the *p53* gene was transferred with 95 - 100% efficiency, as detected by immunohistochemical analysis, when the cells were infected with Ad5CMV-*p53* at a multiplicity of infection of thirty to fifty plaque-forming units (PFU)/cell. Western blots showed that the *p53* protein was expressed at a high level in these cells. Growth of the lung cancer cells with *p53* deletion or mutation was greatly inhibited by Ad5CMV-*p53*, while that of the cell line containing wild-type *p53* was less affected. Tumorigenicity tests in nude mice demonstrated that Ad5CMV-*p53* prevented tumour formation [99]. Induction of apoptosis was shown to be one of the mechanisms for the tumouricidal effect of Ad5CMV-*p53* [100]. The Ad-mediated *p53* gene transfer has also been demonstrated to be effective in suppressing tumorigenicity in different animal models [101,102]. These results suggest that adenovirus is an efficient vector for mediating transfer and expression of tumour suppressor genes in human cancer cells and that the *p53* Ad may be further developed into an effective therapeutic agent in cancer. Clinical trials of Ad-mediated *p53* gene transfer for treatment of lung, head and neck, and liver cancers are underway.

### Toxin or prodrug-activation gene therapy

Toxin gene transfer into cancer cells represents a straightforward approach in cancer gene therapy. The therapeutic index will be largely dependent on the specificity of gene delivery and efficiency of gene transfer *in vivo* to target tumour cells. Genetic prodrug-activation therapy (GPAT) represents a selective genetic strategy against cancer. This involves delivery of a prodrug activating enzyme gene into both tumour and normal cells. By linking the enzyme gene downstream of tumour-specific transcription units, tumour-specific prodrug activation can be achieved. For a detailed review on the prodrug-activation genes for selective cancer chemotherapy, reference is made to a recent article in *Cancer Gene Therapy* [103].

### Toxin gene therapy

Cell killing by expression of the diphtheria toxin A chain coding sequence has been demonstrated *in vitro* [104] and in animals [105,106]. Diphtheria toxin is an extremely potent inhibitor of protein synthesis in eukaryotic cells. It has been estimated that one molecule of diphtheria toxin A per cell is sufficient to kill murine L cells [107]. The toxin is composed of two subunits. The B chain (342 amino acids) is adsorbed to the cell surface for internalisation and the A chain (193 amino acids) specifically modifies histidine residues of elongation factor 2 by ADP-ribosylation, which prevents protein synthesis and kills the cell. Expression of the toxin may be induced by linking the diphtheria toxin A coding sequence with tissue-specific transcription regulatory elements (promoters and enhancers). This approach has been tested in B-lymphoid cells, in which a plasmid was transfected that contained the diphtheria toxin A chain coding sequence under control of the engineered immunoglobulin kappa light chain gene regulatory sequences [108]. This construct specifically expressed diphtheria toxin A in mature B-cells but not in pre-B-cells, suggesting that the construct may be further developed to allow therapeutic ablation of malignant B-cells of mature stages while sparing normal progenitor cells.

A similar method used *Pseudomonas* exotoxin, which was conjugated with IL-4 to treat murine sarcoma and colon adenocarcinoma cells that express high-affinity IL-4 receptors [109]. The chimeric IL-4 *Pseudomonas* exotoxin protein was shown to be cytotoxic to the tumour cells by inhibiting cellular protein synthesis in a dose-dependent manner. A nonchimeric *Pseudomonas* exotoxin protein that could not bind to the IL-4 receptor did not inhibit protein synthesis in tumour cells. A chimeric mutant protein that could bind to IL-4 receptor but did not have the capacity to inhibit protein synthesis was not cytotoxic to tumour cells. The protein synthesis-inhibitory activity of the IL-4/*Pseudomonas* exotoxin fusion protein could be completely abolished by a neutralising antibody to IL-4. These data suggest that a receptor-mediated toxin therapy might be effective.

A new approach in the technology of toxin gene therapy is transfer of the E1A gene of the type-5 adenovirus into tumours. The E1A protein has been shown to be able to induce apoptosis [110,111]. This protein was also shown to suppress expression of the *HER-2/neu* proto-oncogene in cancer cells [112]. The frequent amplification or overexpression of the *HER-2/neu* gene, observed in different types of human cancer, has been shown to correlate with shorter survival time or lower survival rate in ovarian cancer patients. Based on these observations, a strategy to use E1A as a therapeutic gene for treatment of ovarian

cancers that overexpress *HER-2/neu* was developed [113].

### Prodrug-activation gene therapy

Prodrug-activation gene therapy is based on the introduction of a drug sensitivity gene into target cells, which are then killed by administration of the drug at doses that are not detrimental to normal cells. One such 'prodrug-activation' gene that has been successfully used to confer drug sensitivity in an animal model system is the herpes simplex virus thymidine kinase gene (*HSV-tk*). The HSV-TK enzyme can specifically catalyse the phosphorylation of a number of nucleoside analogues, such as acyclovir or ganciclovir, which are poor substrates for the TK enzymes of mammalian cells [114]. The phosphorylated acyclic nucleoside becomes active when incorporated into newly synthesised DNA, resulting in a cytotoxic effect by induction of DNA strand breaks and inhibition of DNA polymerase activity [115].

In a syngeneic mouse model, subcutaneous tumours developed from the *HSV-tk*-gene transduced tumour cells went into complete regression following intraperitoneal administration of ganciclovir, while the tumours derived from the nontransduced tumour cells were not affected [116]. An analogous approach has been taken for treatment of brain tumours in a rat model [117]. In these experiments, the *HSV-tk* retrovirus-producing cells were stereotactically injected into rat cerebral gliomas *in vivo*. The *HSV-tk* retroviruses generated from the producer cells were expected to infect the proliferating tumour cells preferentially, which would then be killed selectively by ganciclovir administered intraperitoneally. Indeed, complete regression of the glioma was observed in eleven of fourteen rats [117]. Since it was unlikely that all of the tumour cells became infected with the *HSV-tk* retroviruses, it was suggested that this regression may have been due to a 'bystander effect'. This effect was demonstrated by a co-culture experiment *in vitro*, in which *HSV-tk* transduced human fibrosarcoma cells induced the ganciclovir-killing effect on non-transduced co-culture cells through a gap junction-mediated metabolic co-operation [118]. Although the mechanism of the bystander effect *in vivo* was not well understood, the remarkable success of this technique in the treatment of a very aggressive tumour that has an extremely poor prognosis when treated with conventional therapy has led to human clinical trials [119].

The therapeutic approach called 'virus-directed enzyme/prodrug therapy' (VDEPT) is another example of prodrug-activation gene therapy [120]. In treatment of hepatocellular carcinoma with VDEPT, the varicella-zoster virus thymidine kinase (*VZV-tk*) gene, that was

transcriptionally regulated by either the hepatoma-associated alpha-fetoprotein or liver-associated albumin promoters, was constructed into a retroviral vector. After infecting the cancer cells with this vector, non-toxic prodrug 6-methoxypurine arabinonucleoside (araM) activated by *VZV-tk* was expressed preferentially in hepatoma cells. The final product, adenine arabinonucleoside triphosphate (araATP), selectively induced cytotoxicity in the hepatoma cells that expressed the gene.

The gene encoding cytosine deaminase can also be used to prime cell death upon administration of a drug that is not normally toxic to eukaryotic cells [121]. Cytosine deaminase converts the nontoxic substance 5-fluorocytosine to a toxic derivative, 5-fluorouracil. Thus, only genetically modified cells carrying and expressing the cytosine deaminase gene are able to synthesise 5-fluorouracil and induce the cytotoxic effect. Retrovirus-mediated cytosine deaminase gene transfer in various cell types has demonstrated this specific cell killing effect after treatment of the transduced cells with 5-fluorocytosine [122].

Recent advances in this technology are improvements in enhancing tumour-killing efficacy and reducing side-effects on normal cells by tissue- or cell-specific expression of prodrug-activation genes. Several recent publications describe in detail the designs and applications of this approach [123-125]. Also, experimental tumour therapy in mice using cyclophosphamide-activating cytochrome P450 2B1 gene transfer into glioma models has been reported to sensitise the tumour cells to the cytotoxic effects of cyclophosphamide [126]. It was suggested that the *in situ* activation of cyclophosphamide by cytochrome P450 2B1 may provide a novel approach for brain tumour gene therapy.

Using radiation to sensitise tumour cells preferentially for gene therapy is a new approach [127]. Transcriptional regulation of the promoter/enhancer region of the *Egr-1* gene can be specifically activated by ionising radiation. A plasmid construct, made by linking the promoter region of *Egr-1* to tumour necrosis factor (TNF)- $\alpha$ , was stably transfected into a human haematopoietic cell line, which exhibited 3.2-fold induction of TNF- $\alpha$  upon radiation at 20 Gy. The radiosensitising *Egr*-TNF cells were injected into human xenografts of the radioresistant squamous cell carcinoma cell line SQ-20B in nude mice. Animals treated with the *Egr*-TNF cells and radiation demonstrated an increase in tumour cures compared with animals treated with radiation alone or unirradiated animals given injections of the cells alone [127].

## Drug-resistance gene therapy

This approach, also called chemoprotection, adopts a strategy that is the opposite of toxin or prodrug-activation gene therapy. It protects drug-sensitive cells, such as bone marrow stem cells, from chemotherapeutic drugs with a drug-resistant gene, which allows the sensitive cells to survive treatment with chemotherapeutic drugs so that a maximal tumouricidal effect can be achieved. A recent review article provides supplemental references for gene transfer of drug resistance genes and implications for cancer therapy [128].

## Multidrug resistance gene and other chemoresistant genes

Chemoresistance is one of the principal obstacles to effective systemic treatment of cancer [129]. The high frequency of a phenomenon called multidrug resistance (MDR), seen both in the clinical treatment of cancer and in tissue culture models, suggests that cancer cells may have specific gene products that confer simultaneous resistance to many different kinds of anticancer drugs. When highly resistant cell clones were selected by sequential multiple drugs [130], a gene, named as *MDR1*, was isolated, and the level of 4.5 kb mRNA in cells correlates with the degree of resistance [131]. The *MDR1* gene was soon shown to encode a 170 kDa membrane glycoprotein known as P-glycoprotein [132]. The predicted structure of the protein includes two groups of six transmembrane domains, with each group containing an ATP binding/utilisation site within a cytoplasmic region. This identifies the P-glycoprotein as a member of the transporter superfamily [133], which also includes the CFTR protein, a sex peptide transport system from yeast, and bacterial nutrient and polypeptide toxin transport systems.

Morphological, biochemical, and physiological studies have shown that the product of the *MDR1* gene is an ATP-dependent multidrug transporter [134]. Three independent lines of investigation have provided evidence that P-glycoprotein is responsible for MDR in many instances:

- the *MDR1* gene is overexpressed in a number of MDR cell lines [132,135];
- transfer and expression of the *MDR1* gene is sufficient to induce MDR in drug-sensitive cells [136,137];
- expression of the human *MDR1* gene in transgenic mice has been shown to result in resistance to drug-induced bone marrow suppression [138].

Identification and characterisation of *MDR1* and its product P-glycoprotein were the key steps that led to understanding of the mechanism of MDR. However, experimental observations have revealed that MDR is a heterologous cellular response to cytotoxic drugs, suggesting that the mechanism of MDR is more complex than may be hypothesised on the basis of the single P-glycoprotein [139]. There are multiresistant cell lines that do not express the P-glycoprotein, indicating that other drug-resistant mechanisms may exist [140]. Examples of proteins that confer drug resistance by some other mechanisms include protein kinases, glutathione-S-transferase, and topoisomerase II.

An increase in the level of protein kinase C (PKC) was observed in the MDR cells of human mammary carcinoma. Treatment with phorbol esters, which activate PKC, increases resistance and reduces the intracellular accumulation of doxorubicin [141]. Analogous results have been shown with AMP-dependent protein kinase A activity. Phosphoprotein changes accompanying the development of resistance to mitomycin C were detected in human colon tumour cells [142].

Glutathione-S-transferase (GST) catalyses the coupling reaction of intracellular electrophiles with L-g-glutamyl-L-cysteinylglycine (GSH). The multifunctional isozymes of GST, which are found in virtually all tissues, are involved mainly in phase II of liver detoxification, in which xenobiotics are inactivated by GST through conjugation. Elevation of GST has been shown to be associated with the acquired resistance of cells to certain anticancer drugs [143]. Further studies showed that the elevated expression of GST correlated with increased resistance to alkylating agents [144,145].

The protein topoisomerase II (Topo II), also known as DNA gyrase, is responsible for the cytotoxicity of anthracycline, acridine, and etoposide, which exert their cytotoxic effect through the formation of a stable ternary complex of DNA-Topo II-drug that alters the processes of DNA duplication and transcription [146]. Different drugs have been shown to alter Topo II activities in different types of cancer [140]. In resistant leukaemia cell lines, the catalytic activity of Topo II was three to five times lower than that of the sensitive parental lines [147].

## Chemoprotection

Multidrug resistance is a major obstacle in cancer chemotherapy. Great research and clinical efforts have been applied to circumvent MDR activities since the MDR genes and their molecular mechanisms were discovered [148]. The two most effective approaches thus far developed are combining chemotherapy either with chemosensitisers that enhance the sensitivity of

target cells to drugs or with immunotherapy that suppresses MDR protein activities. The consideration of MDR as a gene therapy application led to a third, very innovative approach, called chemoprotection. This approach exploits the MDR genes selectively to protect drug-sensitive normal tissues such as bone marrow. After transfer of one of these genes, dosages of anticancer drugs can be raised high enough to kill cancer cells by overcoming their drug resistance.

This approach originated from work in the *MDR1* transgenic mouse model [138]. The high levels of *MDR1* gene products expressed in the transgenic mice successfully protected the sensitive bone marrow from cytotoxic drugs. This effect was confirmed by transplantation of bone marrow from the transgenic *MDR1* mice to drug-sensitive mice, which conferred drug resistance on the recipient animal [149]. These results suggested that it might be possible to introduce the *MDR1* gene into bone marrow *ex vivo*, making that bone marrow resistant to many drugs, and return it to the patient to increase tolerance of and response to high-dose chemotherapy. That expression of the transferred *MDR1* gene is stable and selective in mouse bone marrow has recently been demonstrated by two groups [150,151]. The *MDR1* gene was also shown to function as a dominant selectable marker *in vivo*, allowing a minority of genetically engineered bone marrow cells to be positively selected and enriched by the administration of cytotoxic drugs such as paclitaxel (Taxol) [151]. Chemoprotection from Taxol was evaluated in mice transplanted with *MDR1*-modified bone marrow cells [152]. These mice showed resistance at doses of Taxol that were lethal to mice not transplanted with the *MDR1*-modified marrow. Taxol-resistant haematopoiesis was sustained through five consecutive transplants with *MDR1*-modified marrow, showing that sufficient numbers of early progenitor cells were modified and repopulated under selection by Taxol. Similar Taxol-resistant effects were also obtained with transplantation of human *MDR1* cDNA-modified marrow cells into mice [153]. The success of chemoprotection in the animal models has led to the design of a clinical trial of chemoprotection against Taxol in advanced-stage human epithelial cancers, such as ovarian cancer, by using a similar approach of *MDR1*-modified autologous bone marrow transplantation.

The dihydrofolate reductase gene was similarly used in a retroviral transfer into murine recipients to protect from methotrexate-induced cytopenia. The study demonstrated that mice transplanted with bone marrow cells infected with a retroviral dihydrofolate reductase expression vector showed improved protection from methotrexate-induced marrow toxicity and longer survival than control mice; however, enrichment of

transduced cells by *in vivo* selection could not be detected [154].

A new approach in treatment of cancer with protection of normal tissues is radioprotection. Overexpression of manganese superoxide (MnSOD) has been postulated as one possible mechanism of radioprotection for haematopoietic cells [155]. In this study, the human *MnSOD* gene constructs in both the sense and antisense orientation were transduced into K562 and A375 cells by retroviral vectors. Results demonstrated that K562 cells transduced with *MnSOD* in the antisense orientation displayed increased sensitivity to irradiation and that, in contrast, A375 cells transduced with the sense *MnSOD* gene displayed increased resistance to irradiation compared to both parental or vector-transduced cells. It was proposed that administration of genetically engineered haematopoietic stem cells transduced with *MnSOD* alone or in combination with other antioxidant enzymes could allow for the purging of tumour cells, using higher-dose irradiation *ex vivo*, and may improve the resistance of bone marrow cells during high-dose radio- or chemotherapy *in vivo* [155].

### Cytokine gene therapy and tumour vaccination

Major progress has been made in the understanding not only of the molecular mechanisms underlying carcinogenesis, but also of the complex relations between cancer and the immune system. Among all of the approaches of gene therapy for cancer, cytokine gene therapy and tumour vaccination have a unique strategy that aims at utilising host immune responses to suppress or eliminate tumour cells, which is particularly important for metastatic cancers. Transfer of some cytokine or costimulatory factor genes has been demonstrated to induce immune responses protecting the animal against subsequent injection of parental tumour cells, and can even, in some cases, treat efficiently animals carrying pre-existing parental tumours. The rapid advances in this methodology formed the basis for the recent elaboration of a fast increasing number of clinical trials using cytokine gene transfer for treatment of cancer.

### Genetic modulation of lymphocytes

It was the genetic modification of lymphocytes by retrovirus-mediated gene transfer that initiated human gene therapy [156,157]. The reasons for choosing lymphocytes as gene-transfer recipients were:

- lymphocytes are readily isolated from patients and cultured *in vitro*.

- tumour-infiltrating lymphocytes were identified as a specific antitumor immunoeffector and their kinetics *in vivo* needed to be studied; and
- ethical and safety considerations required that retrovirus-mediated gene transfer first be tested through an *ex vivo* approach.

The clinical trials in which lymphocytes were used to carry a marker or therapeutic gene actually started the *ex vivo* gene transfer technology that is now very useful in various cancer gene therapies, such as gene-modified autologous tumour cell vaccination.

#### **Gene marking in tumour-infiltrating lymphocytes**

Tumour-infiltrating lymphocytes (TIL) are lymphoid cells that accumulate in tumour masses. TIL are isolated from resected tumours by incubation of the heterogeneous single cell suspension derived from tumours with IL-2 [158,159]. Some TIL have the ability to recognise antigens associated with autologous tumour and to kill tumour cells, as well as secrete cytokines [160,161]. In one animal model in which lymphokine-activated killer cells (LAK) are relatively ineffective, TIL were shown to mediate the regression of established tumour deposits with a potency fifty to one hundred times that of LAK cells [158]. In clinical trials, it was possible to grow TIL from approximately 50% of the tumours, and these TIL kill autologous tumour cells from approximately one-third of patients with melanoma [159,162]. Studies of the distribution of TIL following intravenous injection demonstrated that TIL could accumulate in tumour deposits [163,164]. Among more than fifty patients with metastatic melanoma who underwent TIL adoptive therapy, 38% demonstrated an objective regression; however, these responses were of short duration [165]. Both animal and clinical data suggested that TIL may be genetically modified to improve their antitumor therapeutic effectiveness.

Gene marking was used in the first phase of the genetic modification of TIL. The cells were transduced with the neomycin-resistant gene (*Neo<sup>R</sup>*) by retrovirus-mediated gene transfer and introduced into humans for study of their long-term distribution and survival [156,166]. The experimental results showed that the *Neo<sup>R</sup>* gene was well expressed and the modified TIL had no detectable changes in their general properties. After the infusion of the gene-modified TIL into patients, the cells were detectable in the circulation for up to 189 days and in tumour deposits for up to sixty-four days. Safety tests performed on samples from the patients who received the gene-modified cells were all negative and no antibodies against the vector retrovirus were detected by western blot assays of patients' serum at varying times up to 180 days after cell infusion [156]. This was the first clinical trial that

used autologous cells as vehicles for retrovirus-mediated gene transfer in man. The publication of this trial had a profound impact on human gene therapy; it provided data that supported the feasibility and safety of using autologous cells as vehicles for retrovirus-mediated gene transfer. Following the example of TIL-gene marking, many other gene marking experiments have been carried out using this *ex vivo* technique. For example, marking autologous marrow with the *Neo<sup>R</sup>* gene was intended to identify whether the cells responsible for relapse were the gene-transferred cells or residual cells in patients [167]. A proposed protocol that would use dual retroviral markers to test the relative contributions of marrow and peripheral blood autologous cells to recovery after reparative therapy for chronic myelogenous leukaemia is expected to be initiated this year [168].

#### **Cytokine gene-modified tumour-infiltrating lymphocytes**

As an extension of the *Neo<sup>R</sup>*-TIL gene-marking study, modification of TIL by addition of the gene for tumour necrosis factor (TNF) has been studied in patients with malignant melanoma. Tumour necrosis factor (TNF) produces very encouraging antitumour responses in mice, but the maximal tolerated dose in humans is about forty-fold less than the doses required to elicit these responses in mice [169]. Toxic effects are common at doses above 8 mg/kg in man, whereas in the mouse 400 mg/kg can be achieved. Therefore, a strategy was developed to deliver TNF effectively to the site of the tumour using TIL as cellular vehicles [170]. The TIL were isolated directly from the tumour and then grown in large numbers in tissue culture with IL-2. After expansion in culture, they were genetically engineered to produce TNF and were given to the patient intravenously along with high doses of IL-2 for several days. The results of this clinical trial are not yet available.

It was proposed that TIL could also be modified with other genes such as those that encode interferons (IFN- $\beta$  or IFN- $\gamma$ ), other cytokines (IL-1 $\alpha$ , IL-6, and IL-7), or receptors (of Fc, chimeric T-cells, or IL-2) [171]. However, TIL have several drawbacks for use in cytokine gene transfer:

- technical difficulties in isolation and culture;
- poor efficiency for gene transduction;
- weak expression of the transduced gene; and
- low percentage of homing (< 0.015% per gram of tumour).

These were demonstrated by a recent comparison of TNF gene expression in TIL *versus* the melanoma cell lines that were derived from the same enzymatically

digested tumour biopsies used for isolating TIL [172]. Therefore, an alternative approach is being pursued, using tumour cells as vehicles to carry cytokines and other immune-modulating genes for systemically boosting the antitumor immune response.

#### **Single chain antibody gene-modified T-lymphocytes**

Since the isolation, expansion, and tumour-homing efficiency of TIL are limited, an experimental approach which exploits the targeted cytolytic activity of lymphocytes is being developed [173]. Two basic designs, composed of either double chimeric T-cell receptor chains (cTCR) or single-chain Fv linked to the signal transducing  $\gamma$  or  $\zeta$  subunits of the FcR or CD3 (scFvR), have been constructed. Both chimeric receptor genes when transfected into human leukaemic T-cells, TIL or peripheral blood lymphocytes (PBL), endow the recipient T-cells with non-MHC restricted, antibody-type specificity [174]. Upon binding to antigen (either immobilised or displayed on the surface of target cells), the chimeric receptors could transmit a signal for T-cell activation. Since this approach consists of modifying T-cells with an antibody gene, it is also called 'T-body' technology.

In this approach, several technical aspects need to be considered:

- Consistent alterations of tumour cell surface antigens are available as targets;
- The genes of the monoclonal antibodies (mAb) against the antigens are cloned;
- A chimeric gene needs to be constructed that encodes a fusion protein of the Fv region of mAb, a linkage fragment, and the transmembrane and cytoplasmic region of the T-cell receptor (TCR)/CD3 (e.g.,  $\zeta$  chain);
- A high-efficient vector to deliver the chimeric gene into T-cells is necessary.

A recent report on this type of technology described generation of CTLs with specificity for ERBB2 receptor-expressing tumour cells [175]. Overexpression of the ERBB2 receptor is frequently observed in human breast and ovarian carcinomas and provides a target at the cell surface which strongly distinguishes tumour cells from their normal counter parts. A binding function was conferred directly on the  $\zeta$  chain of the TCR complex to circumvent major histocompatibility complex-restricted antigen recognition through the  $\alpha$  and  $\beta$  chains of the TCR. A chimeric gene was constructed which encoded a single-chain Fv antibody (scFv, consisting of the joined heavy- and light-chain variable domains of a monoclonal antibody against the extracellular domain of the ERBB2 receptor), a hinge region as a spacer, and the  $\zeta$  chain of the TCR. This

gene was introduced into CTLs by retroviral gene transfer. The signalling potential of the scFv/hinge/ $\zeta$  receptors was demonstrated by secretion of interferon  $\gamma$  upon coinoculation with ERBB2-expressing cells. Target cells expressing the ERBB2 gene were lysed *in vitro* with high specificity by the scFv/hinge/ $\zeta$ -expressing T-cells. The growth of ERBB2-transformed cells in athymic nude mice was retarded by adoptively transferred scFv/hinge/ $\zeta$ -expressing CTLs. Transduced CTLs labelled with a fluorescent dye were specifically detected in tumour sections. These results suggest that tumour cell lysis by CTLs grafted *in vitro* with major histocompatibility complex-independent recognition could become a gene-therapy approach to cancer treatment.

#### **Cytokine gene-modified tumour cell vaccination**

The use of autologous tumour cells as vaccines to augment antitumor immunity has been explored throughout this century [176]. Although only a few tumour-specific surface antigens, which can be recognised by effector killer cells such as T-cells, have been identified, local cytokine production is believed to play an important role in invoking tumour immunity [177-179]. Studies have shown that transfer of a cytokine gene into tumour cells leads to continuous local delivery of the cytokine. In these studies, local secretion of cytokines by gene-modified tumour cells not only stopped tumour growth but also, in certain murine models, induced a specific immunity to subsequent tumour challenge. In some cases, immunisation with gene-modified tumour vaccines resulted in regression of existing parental tumours. With the evidence obtained from the individual cytokine studies described herein, a firm basis can be laid for proposing that production of new tumour vaccines by gene transfer or genetic modulation will be a powerful tool for enhancing antitumour immunity. It is also feasible that combinations of these cytokines in genetic modification of tumour vaccines will be even more effective, representing one of the most promising approaches in the development of cancer gene therapy. For further references on experimental and clinical studies of cytokine gene-modified tumour cells, please refer to a recent detailed review in *Human Gene Therapy* [180].

#### **Interleukin-4**

Interleukin-4 (IL-4) is produced by the Th2 subset of activated T-helper cells as well as by mast cells. IL-4 participates in the regulation of growth and differentiation of B-cells and T-cells and the generation of cytotoxic T-lymphocytes (CTL). It also activates microvascular endothelium and induces upregulation of vascular cell adhesion molecule 1, which potentiates extravasation of lymphocytes and monocytes from the



Further experimental data on *IFN-γ*-mediated tumour vaccination demonstrated that transduction of human melanoma cells with *IFN-γ* gene enhanced cellular immunity [202]. The *IFN-γ*-transduced and corresponding parental melanoma cells were used for the induction of short-term lymphocyte cultures. Peripheral blood lymphocytes or lymph node cells from twenty melanoma patients were stimulated for five to fifteen days with autologous or MHC class I-matched allogeneic parental or *IFN-γ*-transduced melanoma cells. Seven of the twenty lymphocyte cultures showed substantial increases in lytic activity following stimulation with the transduced melanoma cells in comparison to control lymphocyte cultures stimulated with unmodified parental melanoma. The cytolytic activity stimulated with *IFN-γ*-modified melanomas was mediated partly by MHC-restricted cytotoxic T-lymphocytes and partly by NK cells. Lymphocyte cultures that displayed increases in cytotoxicity after stimulation with the *IFN-γ*-transduced melanoma cells also exhibited enhanced expression or induction of one or more of the following lymphokines: IL-4, IL-1 $\alpha$ , IL-1 $\beta$ , *IFN-γ*, and TNF- $\alpha$ . These studies led to a clinical trial of human *IFN-γ*-transduced autologous tumour cells in patients with disseminated malignant melanoma.

#### **Tumour necrosis factor alpha**

Tumour necrosis factor alpha (TNF- $\alpha$ ) is a potent immunomodulatory molecule affecting the function of many cells involved in the immune response and tumour vasculature, including T-cells, B-cells, neutrophils, monocytes, and macrophages. TNF- $\alpha$  induces MHC class I and class II molecules [203]. Tumour necrosis factor (TNF) was originally described as an antitumor agent *in vivo*, and it has been demonstrated that injection of recombinant TNF- $\alpha$  can mediate the necrosis and regression of a variety of established murine tumours [204,205]. However, the biological effects of TNF *in vivo* depend on its concentration. At an optimal concentration, TNF produces beneficial effects such as cytotoxic and antitumor effects, while higher concentrations of TNF induce harmful effects such as catastrophic tissue injury, organ failure, and irreversible shock leading to death [206]. Although the exact mechanisms of the *in vivo* effects of TNF- $\alpha$  are not known, it appears to have a significant enhancement on CD8<sup>+</sup> T-cells.

The TNF- $\alpha$  gene can be successfully introduced into murine and human tumour cells by retroviral vectors. Weakly immunogenic tumour cells modified by the TNF- $\alpha$  gene grew slowly *in vitro* and were found to regress after an initial phase of growth. This tumour regression was abrogated by depletion of CD4<sup>+</sup> and CD8<sup>+</sup> subsets *in vivo*, suggesting that these T-cells are involved in the immune response [207]. In a separate

study, macrophages were observed at the sites of murine plasmacytomas that were engineered to secrete TNF- $\alpha$ , implicating inflammatory cells in the rejection of this tumour [208]. However, nonimmunogenic murine fibrosarcoma cells that produced TNF grew progressively in syngeneic mice, although IL-2 was effective in inhibiting this growth [209]. Clinical trials involving immunisation of melanoma patients with autologous TNF-producing tumour cells are in progress.

#### **Interleukin-7**

Interleukin-7 (IL-7) was initially described as a growth factor for B-cell progenitors; however, this protein has been demonstrated to stimulate the growth of mature CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, inducing LAK activity [210]. After stimulating T-cells *via* an antibody against T-cell receptor, an IL-7-dependent, IL-2-independent proliferative pathway has been identified, suggesting that IL-7 may function in the absence of IL-2 to regulate T-cell proliferation. Moreover, IL-7 is capable of inducing tumouricidal activity by peripheral blood monocytes [211].

Murine plasmacytoma cells producing IL-7 were completely rejected after injection into mice. The immune response was primarily mediated by CD4<sup>+</sup> T-cells [212]. However, an IL-7-transduced murine fibrosarcoma was heavily infiltrated with CD8<sup>+</sup> T-cells that were believed to be responsible for the slow growth and tumour regression [213]. Antitumour CTL, generated by drainage of lymph node cells and culture with IL-7, are effective in treating three-day pulmonary metastases of syngeneic methylcholanthrene-induced sarcoma in murine models. Furthermore, systemic administration of IL-2 was synergistic with IL-7-expanded cells in this model, but IL-7 was not. Interleukin-7 is the only cytokine other than IL-2 that has been shown to expand therapeutically effective lymphocytes when adoptively transferred to tumour-bearing mice [214]. Clinical trials on IL-7 gene therapy for patients with metastatic colon cancer, renal cell cancer, malignant melanoma or lymphoma are ongoing.

#### **Granulocyte-macrophage colony-stimulating factor**

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a potent factor that is most often associated with the growth and differentiation of haematopoietic progenitors. Several reports suggest that GM-CSF plays an important role in the maturation and/or function of specialised antigen presenting cells [215]. Incubation of GM-CSF in mouse marrow cultures generated large numbers of dendritic cells, which are potent antigen-presenting cells [216].

In a recent study, vaccination of mice with irradiated tumour cells engineered to secrete murine GM-CSF

was demonstrated to stimulate potent, specific, and long-lasting antitumour immunity [217]. When this murine GM-CSF tumour vaccination model was compared to other cytokine-transduced tumours by the same retroviral vector, it was shown that GM-CSF was the most potent stimulator of systemic antitumor immunity among the ten proteins tested, including IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN- $\gamma$ , IL-1RA, (ICAM), (CD2), and TNF- $\alpha$  [217]. The success of immunisation with GM-CSF-transduced tumours was dependent on both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, despite the fact that the tumours were MHC class II negative. The potency of GM-CSF's effect locally might relate to its unique ability to promote the differentiation of haematopoietic precursors to dendritic cells, which could specifically enhance tumour-antigen presentation. Clinical trials of GM-CSF gene mediated tumour vaccination for prostate and renal cell cancers have begun.

#### **Granulocyte colony-stimulating factor**

Granulocyte colony-stimulating factor (G-CSF) is characterised as a potent differentiation-inducing factor, essentially in granulopoiesis [218]. Murine colon adenocarcinoma cells expressing G-CSF were rejected from syngeneic mice or *nu/nu* mice. Histologic examinations demonstrated that the antitumour effect was associated with a massive infiltration of neutrophilic granulocytes [219,220]. In contrast, G-CSF tumour vaccines were unable to eradicate an already established G-CSF-secreting carcinoma without the aid of T-lymphocytes [221].

The results of studies that compared the cytokine genes in different tumour cells suggested that the effect of immunomodulation by tumour engineering might depend on both the responsiveness of host cell types and the immunogenicity of tumour cells. Although the results varied in the different tumour systems, because of differences in cell dose, type of cytokine, expression level of transduced gene, and location of immunisation, the effectiveness of autologous tumour vaccines has been well established. The success of the cytokine gene-modified tumour vaccines is believed to depend on a paracrine mechanism in which a locally high concentration of cytokines induces an antitumour immune response, which can be either specific or non-specific. High concentrations of cytokines often induce a non-specific local inflammatory response, which causes the injected tumour cells to be eliminated and immunisation to fail. An alternative in autologous tumour vaccine strategy is to modulate tumour cell antigen presentation by transferring genes such as MHC, B7, and others. This approach may avoid the cytokine-induced local inflammatory response while still inducing local immunisation.

#### **Antigen presentation gene modulation for tumour cell vaccination**

Many tumour cells have been shown to carry tumour antigens or tumour-associated peptides that should be capable of activating host T-cells. However, secondary cell-surface changes as well as effects of immunosuppressive factors may render them defective in antigen presentation and inadequate in activation of tumour-specific T-lymphocytes. Several studies in which tumour-specific T-lymphocyte activity was amplified have shown that such amplification can result in potent tumour-specific immunity that can mediate tumour rejection in the autologous host [187,222,223]. Experimental studies have defined a key role for T-lymphocytes in rejection of these tumours, and both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells have been implicated in tumour rejection [224]. Activation of CD4<sup>+</sup> T-cells is thought to require two signals from the antigen-presenting cell (APC). The first signal is the engagement of the T-cell receptor (TCR) for the antigen by the MHC II/peptide complex of the APC [225]. The second signal is thought to be the interaction of the B7 co-stimulatory molecules of the APC with its cognate receptors, CD28 and/or CTLA-4, on the responding T-cell [226-229]. The delivery of the two signals has been shown to be linked and to be connected with the engagement of the class II/peptide complex by the TCR-initiated intracellular signalling events within the APC that stimulated upregulation of B7 on the APC surface [230].

It has long been known that MHC molecules, both class I and class II, are involved in tumour antigen presentation, but through different pathways. Class I MHC can enhance activation of tumour-specific CTL *in vitro*. Early work on tumour vaccination that used transfection of MHC class I genes resulted in suppression of the tumour cells in tumorigenicity and/or metastasis in mouse models [231,232]. MHC class II genes were shown to be involved in activation of tumour-specific T-helper cells, and their introduction into tumour cells resulted in a decrease in the tumorigenicity and generated a systemic immune response against the parental tumour [222]. Despite these positive results, the relationship between levels of MHC expression and immunogenicity is inconsistent among tumour models. Researchers have recently begun to believe that the inconsistency is caused by other cofactors, such as the B7 co-stimulatory molecule, which affects the antigen presentation by MHC/peptide complexes.

The molecule B7 is normally expressed as an activation antigen on antigen-presenting cells such as B-cells, macrophages, and dendritic cells. There are two distinct B7 molecules: B7-1 is the ligand for CD28 and CTLA4, and B7-2 is an alternative ligand for CTLA4 [233,234]. Although the function of CTLA-4 (expressed



exclusively on CD8<sup>+</sup> T-cells) is as yet unknown, much evidence has accumulated that CD28 (expressed on all CD4<sup>+</sup> and most CD8<sup>+</sup> T-cells) is a critical receptor for co-stimulatory signals in T-cell activation. Since CD28 has been shown not only to enhance the level of lymphokine production by CD4<sup>+</sup> T-cells subsequent to T-cell receptor engagement but also to promote engagement of CD8<sup>+</sup> cells for CTL priming or activation, it is rational to select the B7 gene for transfer into tumour cells to enhance their immunogenicity.

A number of laboratories have reported that, after transfection with a B7 gene, autologous tumours were rejected in their syngeneic host. In addition, systemic immune responses were induced which abrogated the parental tumours at a distant site [235-238]. B7 showed different potency in different types of tumours and animal models. Expression of the co-stimulatory ligand B7 on melanoma cells was found to induce the rejection of a murine melanoma *in vivo*. This rejection was mediated by CD8<sup>+</sup> T-cells; CD4<sup>+</sup> T-cells were not required. [236]. When mouse SaI sarcoma cells that bore a truncated MHC II molecule and were transfected with B7 cDNA were injected into the mice, they became resistant to challenges of wild-type MHC II<sup>+</sup>B7<sup>+</sup> ascites or solid SaI tumour; the induced immunity required CD4<sup>+</sup> T-cells and was specific for the immunising sarcoma cells [237]. In another case, the transduction of B7 into a tumour by itself was insufficient to cause rejection or systemic immune response; it was necessary to introduce an additional 'strong' tumour antigen into the tumours to obtain the B7-enhanced tumour vaccine effect [238]. In this study, the transfection of B7 into MHC I<sup>+</sup>/II<sup>+</sup> tumour cells did not enhance their immunogenicity, but the co-introduction of the B7 and MHC II genes did. Although application of B7 to modify tumours is not the final answer to tumour vaccine design, these studies have opened up a novel approach to construction of artificial tumour antigens and their presentation to T-cells as part of the generation of a systemic antitumour immune response.

### Combinational gene therapy

This type of methodology seeks to achieve increased efficacy of treatment through the additive or synergistic effects of two or more approaches of cancer gene therapy or gene therapy with conventional cancer therapy. The early report on the strategy of combinational therapy was of tumour suppressor gene therapy combined with chemotherapy. The p53 protein is an inducer of apoptosis, as is the chemotherapeutic agent Cisplatin. When tumours of animal models were treated locally with Ad5CMV-p53 and systemically with Cisplatin, a stronger tumouricidal effect with local

massive apoptosis of tumour cells was detected [239]. The possible synergistic mechanism underlying this effect may be that the DNA damage caused by Cisplatin in tumour cells that were infected with Ad5CMV-p53 enhanced the proportion of cells entering the p53-dependent apoptotic pathway [240]. This approach has been incorporated into the clinical trial of Ad5CMV-p53 in lung cancer gene therapy, which will be carried out in 1995 at the University of Texas MD Anderson Cancer Center.

Another strategy for combinational therapy is to express antisense RNA to block the E6 and E7 oncoproteins of human papilloma virus (HPV) and sense RNA for supplementation of tumour suppressor gene *Rb* in cervical cancer cells [241]. The HPV E6 and E7 proteins bind to and inactivate the products of the p53 and *Rb* genes, which contribute to the carcinogenesis of the cervix and are responsible for the neoplastic phenotype of cervical carcinoma. Co-transfection of the plasmids that carry the E6 and E7 genes in antisense orientation and the *Rb* cDNA in sense orientation into HeLa cells inhibited the cell growth *in vitro* and suppressed tumorigenicity in mouse subcutaneous model [241]. This strategy was designed to reverse the process of HPV-mediated cervical carcinogenesis through suppressing expression of the E6 and E7 genes and restoring the function of the *Rb* gene for cell growth control. The efficacy of this approach in cervical cancer treatment needs to be evaluated further.

In attempts to develop more effective tumour vaccines, transfer of two or more cytokine genes into tumour cells may be an option. Gene therapy experiments have been performed on gene therapy for Lewis lung carcinoma (LLC) [242]. *TNF* and *IL-2* cDNAs were introduced into pBMG-Neo and pcDV-x819 vectors, respectively, and then cotransfected into LLC cells. The co-transfectants were selected by incubating them in a medium containing G418 followed by limiting dilution to obtain *IL2* and *TNF* co-transfected LLC (LLC-TNF-IL-2) cells. When  $5 \times 10^5$ /ml LLC-TNF-IL-2 cells were incubated for forty-eight hours, they secreted 7.56 U/ml *TNF* and 527.0 U/ml *IL-2* into the culture supernatant. When C57BL/6 mice were transplanted with  $1 \times 10^6$  LLC-TNF-IL-2 cells, all the tumours were rejected. The growth of transplanted LLC, but not B16F10 melanoma cells, was retarded in mice inoculated with LLC-TNF-IL-2 on their contralateral sides, which suggested specific immunity was induced. The immunisation effect by the co-transfectant was superior to that of the *IL-2*- and *TNF*-transfectants alone.

Recently, there have been publications on combinational gene therapies in which mouse cancer models were treated with local cell killing by HSV-*tk*/ganci-

clovir (HSV-*tk*/GCV) system plus induction of anti-tumour immunity by virus-mediated co-expression of IL-2 or GM-CSF [243,244]. In these experiments, suppression of tumourigenicity by using combinational treatment was significantly stronger than using either approach alone. In addition, systemic anti-tumour immune responses were developed in this combinational gene therapy, which was effective against challenges of tumourigenic doses of parental tumour cells inoculated at distant sites. These results suggest that combination of prodrug-activation and cytokine gene therapies *in vivo* can be a powerful approach for treatment of metastatic cancers.

Combinational interventions among different approaches of gene therapies or through gene therapy with conventional therapies are at an early stage. The rationale for the use of combinational therapy is primarily to improve the effectiveness of a single method that is currently not sufficient to fulfill the therapeutic purpose. Generally, before any specific approach of cancer gene therapy with high efficacy is well developed, combinational therapies will be a useful application in cancer treatment.

### Future prospects

Dynamic development with diversified technical approaches is the current status of the field of cancer gene therapy; this makes its definition rather difficult. Generally, cancer gene therapy uses genetic modulation of cancer cells or anticancer immunity to suppress malignancy through targeted delivery of therapeutic or regulatory genes or genetic therapeutic agents. The diversity of the field indicates that there may be many ways to achieve an anticancer effect through different genetic manipulations. Nevertheless, it also reflects that there is as yet, no consensus on a dominant approach for any given condition. Every approach yet tried has its own strengths and weaknesses. The field is still very young but does have a bright future.

Several technical challenges now require focused attention with intensive research effort if development of cancer gene therapy is to be accelerated. These include:

- The development of better *in vivo* delivery systems with higher therapeutic indexes, focusing on larger gene-carrying capacity, targeted gene delivery, and more efficient gene transfer with less immunogenicity and low cytotoxicity.
- The controllable transgene expression in target cells: on/off switch, sustained, or inducible tissue-specific expression, etc.
- The comparative study of tumour vaccines, which will clarify the relative potency and specificity of cytokines, allowing construction of combined tumour vaccines with specificity to different tumour types.
- The enhancement of tumour antigen presentation by modulating tumour cells with multiple genes such as MHC, B7, and those for specific tumour targeting.
- The development of novel genetic approaches to the prevention of cancer.

We now realise that no single approach to gene therapy will suffice; rather, we need to develop several complementary strategies that can be combined in various ways to treat a given genetic disease effectively. This is particularly true in cancer gene therapy, since cancer is a disease developed through a multistep process of multiple cytogenetic abnormalities. Gene therapy is unlikely to replace conventional cancer therapy, but rather provides an alternative approach. In the near future, combinational approaches among gene therapies or gene therapy with conventional therapies such as chemotherapy, immunotherapy, radiotherapy, and surgery will be widely used, perhaps leading to the development of a more advanced biological therapy for cancer.

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### References

Papers of special interest have been highlighted as:

- of interest
  - of considerable interest
1. HARRIS CC: **Molecular basis of multistage carcinogenesis.** In: *Multistage Carcinogenesis*. Harris CC, Hirohashi S, Ito N, Piat HC, Sugimura T, Terada M, Yokota J (Eds.). Japan Sci. Soc. Press, Tokyo/CRC Press, Boca Raton (1992):3-19.
  2. WEINBERG RA: **Oncogenes, tumour suppressor genes, and cell transformation: trying to put it all together.** In: *Origins of Human Cancer: A Comprehensive Review*. Brugge J, Curran T, Harlow ED, and McCormick F (Eds.). Cold Spring Harbor Laboratory Press, New York (1991):1-16.
  3. STEIN CA, COHEN JS: **Oligodeoxynucleotides as inhibitors of gene expression: a review.** *Cancer Res.* (1988) 48:2659-2668.
- A detailed review of oligodeoxynucleotides as inhibitors of gene expression.
4. HANVEY JC, PEPPER NJ, BISI JE, THOMPSON SA, CADILLA R, JOSEY JA, RICCA DJ, HASSMAN CF, BONHAM MA, AUGER KG, CARTER SG, BRUCKENSTEIN DA, BOYD AL, NOBLE

- SA. BABISS LE: Antisense and antigenic properties of peptide nucleic acids. *Science* (1992) 258:1481-1485.
5. CHOO Y, SANCHEZ-GARCIA I, KLUNG A: *In vitro* repression by a site-specific DNA-binding protein designed against an oncogenic sequence. *Nature* (1994) 372:642-645.
6. MERCOLA D, COHEN JS: Antisense approaches to cancer gene therapy. *Cancer Gene Ther.* (1995) 2:47-59.  
A recent review on antisense approaches to cancer gene therapy.
7. CROOKE ST: Oligonucleotide therapeutics: a prospectus. *Antisense Res. Develop.* (1993) 3:1-2.
8. WICKSTROM E: Oligodeoxynucleotide stability in subcellular extracts and culture media. *J. Biochem. Biophys. Methods* (1986) 13:97-102.
9. HELENE C: Rational design of sequence-specific oncogene inhibitors based on antisense and antigenic oligonucleotides. *Eur. J. Cancer* (1991) 27:1466-1471.  
A good review about design of sequence-specific oncogene inhibitors based on antisense and antigenic oligonucleotides.
10. CROOKE RM: *In vitro* toxicology and pharmacokinetics of antisense oligonucleotides. *Anti-Cancer Drug Design* (1991) 6:609-646.
11. PROCHOWNIK EV: Antisense approaches to assessing oncogene signaling pathway. In: *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York (1992) 1:303-316.
12. STEIN CA, CHENG YC: Antisense oligonucleotides as therapeutic agents - is the bullet really magical? *Science* (1993) 261:1004-1012.  
A good discussion on the basic science and application of oligos as therapeutic agents.
13. SZCZYLIK C, SKORSKI T, NICOLAIDES NC, MANZELLA L, MALAGUARNERA L, VENTURELLI D, GEWIRTZ AM, CALABRETTA B: Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides. *Science* (1991) 253:562-565.
14. SKORSKI T, NIEBOROWSKASKORSKA M, BARLETTA C, MALAGUARNERA L, SZCZYLIK C, CHEN ST, LANGE B, CALABRETTA B: Highly efficient elimination of Philadelphia leukemia cells by exposure to bcr/abl antisense oligodeoxynucleotides combined with Mafosfamide. *J. Clin. Invest.* (1993) 9:194-202.
15. AKHTAR S, IVINSON AJ: Therapies that make sense. *Nature Genet.* (1993) 4:215-217.
16. BAYEVER E, IVERSEN P, SMITH L, SPINOLO J, ZONG G: Guest editorial: systemic human antisense therapy begins. *Antisense Res. Dev.* (1992) 2:109-110.
17. SMITH LJ, MCCULLOCH EA, BENCHIMOL S: Expression of the p53 oncogene in acute myeloblastic leukemia. *J. Exp. Med.* (1986) 164:71-76.
18. MAHER LJ, DOLNICK BJ: Specific hybridization arrest of dihydrofolate reductase mRNA *in vitro* using anti-sense RNA or anti-sense oligonucleotides. *Arch. Biochem. Biophys.* (1987) 253:214-220.
19. WALDER RY, WALDER JA: Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA* (1988) 85:5011-5015.
20. LOKE SL, STEIN C, ZHANG XH, MORI K, NAKANISHI M, SUBASINGHE C, COHEN JS, NECKERS LM: Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA* (1989) 86:3474-3478.
21. YAKUBOV LA, DEEVA EA, ZARYTOVA VF, IVANOVA EM, RYTE AS, YURCHENKO LV, VLASSOV VV: Mechanism of oligonucleotide uptake by cells: involvement of receptors? *Proc. Natl. Acad. Sci. USA* (1989) 86:6454-6458.
22. STEIN CA, TONKINSON JL, ZHANG LM, YAKUBOV L, GERVASONI J, TAUB R, ROSENBERG SA: Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells. *Biochemistry* (1993) 32:4855-4861.
23. AKHTAR S, SHOJI Y, JULIANO RL: Pharmaceutical aspects of the biological stability and membrane transport characteristics of antisense. In: *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York (1992) 1:133-145.
24. SHAW JP, KENT K, BIRD J, FISHBACK J, FROEHLER B: Modified deoxyoligonucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res.* (1991) 19:747-750.
25. LETSINGER RL, ZHANG G, SUN DK, IKEUCHI T, SARIN PS: Cholesteryl-conjugated oligonucleotides: synthesis, properties and activity as inhibitors of replication of human immunodeficiency virus in culture. *Proc. Natl. Acad. Sci. USA* (1989) 86:6553-6556.
26. CLARENCE JP, DEGOLS G, LEONETTI JP, MILHAUD P, LEBLEU B: Delivery of antisense oligonucleotides by poly (L-lysine) conjugation and liposome encapsulation. *Anti-Cancer Drug Design* (1993) 8:81-94.
27. LEONETTI JP, MACHY P, DEGOLS G, LEBLEU B, LESERMAN L: Antibody-targeted liposomes containing oligodeoxynucleotides complementary to viral RNA selectively inhibit viral replication. *Proc. Natl. Acad. Sci. USA* (1990) 87:2448-2451.
28. MILLIGAN JF, JONES RJ, FROEHLER BC, MATTEUCCI MD: Development of antisense therapeutics: Implications for cancer gene therapy. *Annals New York Acad. Sci.* (1994) 716:228-241.
29. EGUCHI Y, ITOH T, TOMIZAWA J: Antisense RNA. *Ann. Rev. Biochem.* (1991) 60:631-652.  
A thorough review on antisense RNA.
30. KRYSTAL GW: Regulation of eukaryotic gene expression by naturally occurring antisense RNA. In: *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York. (1992) 1:11-20.
31. INOLYE M: Antisense RNA: its function and applications in gene regulation - A review. *Gene* (1988) 72:25-34.  
A review of antisense RNA function and its applications in gene regulation.
32. WEINTRAUB HM: Antisense RNA and DNA. *Sci. Am.* (1990) 262:40-48.
33. LEDWITH BJ, MANAM S, KRAYNAK AR, NICHOLS WW, BRADLEY MO: Antisense-fos RNA causes partial reversal of the transformation phenotypes induced by the c-Ha-ras oncogene. *Mol. Cell. Biol.* (1990) 10:1545-1555.
34. SKLAR MD, THOMPSON E, WELSH MJ, LIEBERT M, HARNEY J, GROSSMAN HB, SMITH M, PROCHOWNIK EV: Depletion of c-myc with specific antisense sequences reverses the transformed phenotype in ras oncogene-transformed NIH 3T3 cells. *Mol. Cell. Biol.* (1991) 11:3699-3710.
35. MUKHOPADHYAY T, TALNSKY M, CAVENDER AC, ROTH JA: Specific inhibition of K-ras expression and tumorigenicity of lung cancer cells by antisense RNA. *Cancer Res.* (1991) 51:1744-1748.

36. ZHANG Y, MUKHOPADHYAY T, DONEHOWER LA, GEORGES RN, ROTH JA: Retroviral vector-mediated transduction of K-ras antisense RNA into human lung cancer cells inhibits expression of the malignant phenotype. *Human Gene Ther.* (1993) 4:451-460.
37. GEORGES RN, MUKHOPADHYAY T, ZHANG Y, YEN N, ROTH JA: Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct. *Cancer Res.* (1993) 53:1743-1746.
38. KIBLER-HERZOG L, KELL B, ZON G, SHINOZUKA K, MILLZAN S, WILSON WD: Sequence-dependent effects in methylphosphonate deoxyribonucleotide double and triple helical complexes. *Nucleic Acids Res.* (1990) 18:3545-3555.
39. MAHER LJ, WOLD B, DERVAN PB: Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation. *Science* (1989) 245:725-730.  
First report of inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation.
40. HANVEY JC, SHIMIZU M, WELLS RD: Site-specific inhibition of *EcoRI* restriction/modification enzymes by a DNA triple helix. *Nucleic Acids Res.* (1990) 18:157-161.
41. COONEY M, CZERNUSZEWICZ G, POSTEL EH, FLENT SJ, HOGAN ME: Site-specific oligonucleotide binding represses transcription of the human *c-myc* gene *in vitro*. *Science* (1988) 241:456-459.  
First report of site-specific oligonucleotide binding represses transcription of the human *c-myc* gene *in vitro*.
42. ORSON FM, THOMAS DW, MCSHAN WM, KESSLER DJ, HOGAN ME: Oligonucleotide inhibition of IL2R $\alpha$  RNA transcription by promoter region collinear triplex formation in lymphocytes. *Nucleic Acids Res.* (1991) 19:3435-3441.
43. GEE JE, MILLER DM: Structure and applications of intermolecular DNA triplexes. *Am. J. Med. Sci.* (1992) 304:366-372.  
A detailed review of structure and applications of intermolecular DNA triplexes.
44. ONO A, TS'O OP, KAN LS: Triplex formation of oligonucleotides containing 2'-O-methylpseudo-isocytidine in substitution for 2'-deoxycytidine. *J. Am. Chem. Soc.* (1991) 113:4032-4033.
45. GAGNOR C, BERTRAND JR, THENET S, LEMAITRE M, MORVAN F, RAYNER B, MALVY C, LEBLEU B, IMBACH JL, PAOLETTI C: Comparative study of  $\alpha$  and  $\beta$  anomeric oligodeoxy-ribonucleotides in hybridization to mRNA and in cell-free translation inhibition. *Nucleic Acids Res.* (1987) 15:10507-10521.
46. HELENE C: Control of gene expression by antisense and antigenic oligonucleotide-intercalator conjugates. In: *Gene Regulation: Biology of Antisense RNA and DNA*. Erickson RP, Izant JG (Eds.). Raven Press Ltd., New York (1992) 1:109-118.
47. KIM SH, CECH TR: Three-dimensional model of the active site of the self-splicing rRNA precursor of Tetrahymena. *Proc. Natl. Acad. Sci. USA* (1987) 84:8788-8792.  
First report on the three-dimensional model of the active site of ribozymes.
48. CECH TR: Ribozymes and their medical implications. *JAMA* (1988) 260:3030-3034.
49. VON AHSEN U, SCHROEDER R: RNA as a catalyst: natural and designed ribozymes. *BioEssays* (1993) 15:299-307.  
A thorough review on RNA as a catalyst: natural and designed ribozymes.
50. KASHANI-SABET M, FUNATO T, TONET J, JIAO L, WANG W, YOSHIDA E, KASHFINN BI, SHITARA T, WU AM, MORENO JG, TRAWEEK ST, AHLERING TE, SCANLON KJ: Reversal of the malignant phenotype by an anti-ras ribozyme. *Antisense Res. Dev.* (1992) 2:3-15.  
A significant report on reversal of the malignant phenotype by an anti-oncogene ribozyme.
51. KOIZUMI M, KAMIYA H, OHTSUKA E: Ribozymes designed to inhibit transformation of NIH3T3 cells by the activated *c-Ha-ras* gene. *Gene* (1992) 117:179-184.
52. SIOLD M, NATVIG JB, FORRE O: Preformed ribozyme destroys tumour necrosis factor mRNA in human cells. *J. Mol. Biol.* (1992) 223:831-835.
53. WEERASINGHE M, LIEM SE, ASAD S, READ SE, JOSHI S: Resistance to human immunodeficiency virus type 1 (HIV-1) infection in human CD4+ lymphocyte-derived cell lines conferred by using retroviral vectors expressing an HIV-1 RNA-specific ribozyme. *J. Virol.* (1991) 65:5531-5534.
54. LU D, CHATTERJEE S, BRAR D, WONG KK, Jr.: Ribozyme-mediated *in vitro* cleavage of transcripts arising from the major transforming genes of human papillomavirus type 16. *Cancer Gene Ther.* (1994) 1:267-277.
55. HARRIS H: How tumour suppressor genes were discovered. *FASEB J.* (1993) 7:978-979.  
A thorough review on discovery of tumour suppressor genes.
56. ANDERSON MJ, STANBRIDGE EJ: Tumour suppressor genes studied by cell hybridization and chromosome transfer. *FASEB J.* (1993) 7:826-833.
57. DONEHOWER LA, HARVEY M, SLAGLE BL, MCARTHUR MJ, MONTGOMERY CA, BUTEL JS, BRADLEY A: Mice deficient for *p53* are developmentally normal but susceptible to spontaneous tumors. *Nature* (1992) 356:215-221.  
First report of the model of *p53* deficient transgenic mouse.
58. WIMAN KG: The retinoblastoma gene: role in cell-cycle control and cell differentiation. *FASEB J.* (1993) 7:841-845.  
A detailed review about the role of *Rb* gene in cell-cycle control and cell differentiation.
59. HUANG HJS, YEE JK, SHEW JY, CHEN PL, BOOKSTEIN R, FRIEDMANN T, LEE EYHP, LEE WH: Suppression of the neoplastic phenotype by replacement of the *Rb* gene in human cancer cells. *Science* (1988) 242:1563-1566.  
A significant report of the tumor suppressor activity of the *Rb* gene.
60. BOOKSTEIN R, SHEW JY, CHEN PL, SCULLY P, LEE WH: Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated *Rb* gene. *Science* (1990) 247:712-715.  
First report of suppression of tumorigenicity by restoring a wild type *Rb* gene.
61. MONTENARH M: Biochemical, immunological, and functional aspects of the growth-suppressor/oncoprotein *p53*. *Crit. Rev. Oncog.* (1992) 3:233-256.  
A thorough review of *p53* in biochemical, immunological, and functional aspects.
62. TOMINAGA O, HAMELIN R, REMVIKOS Y, SALMON RJ, THOMAS G: *p53* from basic research to clinical applications. *Crit. Rev. Oncog.* (1992) 3:257-282.
63. MERCER WE: Cell-cycle regulation and the *p53* tumour suppressor protein. *Crit. Rev. Eukaryot. Gene Expr.* (1992) 2:251-263.

64. KLERBITZ SJ, PLUNKETT BS, WALSH WV, KASTAN MB: Wild-type p53 is a cell-cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA* (1992) 89:7491-7495.
65. FIELDS S, JANG SJ: Presence of a potent transcription-activating sequence in the p53 protein. *Science* (1990) 249:1046-1049.  
First report of presence of a potent transcription-activating sequence in the p53 protein.
66. MIETZ JA, UNGER T, HUIBREGTSE JM, HOWLEY PM: The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J.* (1992) 11:5013-5020.
67. WILCOCK D, LANE DP: Localization of p53, retinoblastoma, and host replication proteins at sites of viral replication in herpes-infected cells. *Nature* (1991) 349:429-431.  
A significant report of involvement of the p53 and RB proteins in DNA replication.
68. BARGONETTI J, FRIEDMANN PN, KERN SE, VOGELSTEIN B, PRIVES C: Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. *Cell* (1991) 65:1083-1091.
69. YONISH-ROUACH E, RESNITZKY D, LOTEM J, SACHS L, KIMCHI A, OREN M: Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature* (1991) 352:345-347.  
First report of the p53 protein as an inducer of apoptosis.
70. SHAW P, BOVEY R, TARDY S, SAHLI R, SORDAT B, COSTA J: Induction of apoptosis by wild-type p53 in a human colon tumour-derived cell line. *Proc. Natl. Acad. Sci. USA* (1992) 89:4495-4499.
71. RYAN JJ, DANIH R, GOTTLIEB CA, CLARKE M: Cell-cycle analysis of p53-induced cell death in murine erythroleukemia cells. *Mol. Cell. Biol.* (1993) 13:711-719.
72. FINLAY CA, HINDS PW, LEVINE AJ: The p53 proto-oncogene can act as a suppressor of transformation. *Cell* (1989) 57:1083-1093.  
A significant report of the p53 protein as a tumour suppressor.
73. BAKER SJ, MARKOWITZ S, FEARSON ER, VILLSON JKV, VOGELSTEIN B: Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* (1990) 249:912-915.  
First report of tumour suppressor activity of the wild-type p53 gene.
74. CHENG J, YEE JK, YEARGIN J, FRIEDMANN T, HAAS M: Suppression of acute lymphoblastic leukemia by the human wild-type p53 gene. *Cancer Res.* (1992) 52:222-226.
75. TAKAHASHI T, CARBONE D, TAKAHASHI T, NAU MM, HIDA T, LINNOILA I, UEDA R, MINNA JD: Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. *Cancer Res.* (1992) 52:2340-2343.
76. QUINLAN DC, DAVIDSON AG, SUMMERS CL, WARDEN HE, DOSHI HM: Accumulation of p53 protein correlates with a poor prognosis in human lung cancer. *Cancer Res.* (1992) 52:4828-4831.
77. HORIO Y, TAKAHASHI T, KUROISHI T, HIBI K, SUYAMA M, NIIMI T: Prognostic significance of p53 mutations and 3P deletions in primary resected non-small cell lung cancer. *Cancer Res.* (1993) 53:1-4.
78. SHRIVASTOVA S, ZOU A, PIROLLO K, BLATTNER S, CHANG E: Germline transmission of a mutated p53 gene in a cancer prone family with Li-Fraumeni syndrome. *Nature* (1990) 348:747-749.
79. HARPER JW, ADAM GR, WEI N, KEYOMARSI K, ELLEDGE SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinase. *Cell* (1993) 75:805-816.  
A significant report of identification of the p21 protein as an inhibitor of G1 cyclin-dependent kinase.
80. EL-DEIRY WS, TOKINO T, VELCULESCU VE, LEVY DB, PARSONS R, TRENT JM, LIN D, MERCER WE, KINZLER KW, VOGELSTEIN B: WAF1, a potential mediator of p53 tumor suppression. *Cell* (1993) 75:817-825.  
First report on WAF1, a potential mediator of p53 tumour suppression.
81. ROEMER K, FRIEDMANN T: Mechanisms of action of the p53 tumour suppressor and prospects for cancer gene therapy by reconstitution of p53 function. *Annals New York Acad. Sci.* (1994) 716:265-280.
82. GOYETTE MC, CHO K, FASCHING CL, LEVY DB, KINZLER KW, PARASKEVA C, VOGELSTEIN B, STANBRIDGE EJ: Progression of colorectal cancer is associated with multiple tumour suppressor gene defects but inhibition of tumorigenicity is accomplished by correction of any single defect via chromosome transfer. *Mol. Cell. Biol.* (1992) 12:1387-1395.
83. LEONE A, FLATOW U, KING CR, SANDEEN MA, MARGULIES MK, LIOTTA LA, STEEG PS: Reduced tumour incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells. *Cell* (1991) 65:25-35.  
A significant report of the tumour suppression activity of nm23.
84. FLORENES VA, AAMDAL S, MYKLEBOST O, MAELANDSMO GM, BRULAND OS, FODSTAD O: Levels of nm23 messenger RNA in metastatic melanomas inverse correlation to disease prognosis. *Cancer Res.* (1992) 52:6088-6091.
85. KAMB A, GRUIS NA, WEAVER-FELDDHAUS J, LIU Q, HARSHMAN K, TAVTIGIAN SV, STOCKERT E, DAY RS III, JOHNSON BE, SKOLNICK MH: A cell cycle regulator potentially involved in genesis of many tumour types. *Science* (1994) 264:436-440.  
First report of p16 and p15 as major tumour suppressors (MTS).
86. NOBORI T, MIURA K, WU DJ, LOIS A, TAKABAYASHI K, CARSON DA: Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* (1994) 368:753-756.
87. MIKI Y, SWENSEN J, SHATTUCK-EIDENS D, FUTREAL PA, HARSHMAN K, TAVTIGIAN S, LIU Q, COCHRAN C, BENNETT LM, DING W, BELL R, ROSENTHAL J, HUSSEY C, TRAN T, MCCLURE M, FRYE C, HATTIER T, PHELPS R, HAUGENSTRANO A, KATCHER H, YAKUMO K, GHOLAMI Z, SHAFER D, STONE S, BAYER S, WRAY C, BOGDEN R, DAYANANTH P, WARD J, TONIN P, NAROD S, BRISTOW PK, NORRIS FH, HELVERING L, MORRISON P, ROSTECK P, LAI M, BARRETT JC, LEWIS C, NEUHAUSEN S, CANNON-ALBRIGHT L, GOLDFAR D, WISEMAN R, KAMB A, SKOLNICK MH: A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* (1994) 266:66-71.  
First report of identification of the BRCA1 gene.
88. HANNON G, BEACH D: p15INK4B is a potential effector of TGF- $\beta$ -induced cell cycle arrest. *Nature* (1994) 371:257-261.
89. MARX J: A challenge to p16 gene as a major tumour suppressor. *Science* (1994) 264:1846.

90. ZHANG S-Y, KLEIN-SZANTO AJP, SAUTER ER, SHAFARENKO M, MITSUNAGA S, NOBORI T, CARSON DA, RIDGE JA, GOODROW TL: Higher frequency of alterations in the *p16/CDKN2* gene in squamous cell carcinoma cell lines than in primary tumours of the head and neck. *Cancer Res.* (1994) 54:5050-5053.
91. JEN J, HARPER W, BIGNER SH, BIGNER DD, PAPADOPOULOS N, MARKOWITZ S, WILLSON JK V, KENZLER KW, VOGELSTEIN B: Deletion of *p16* and *p15* genes in brain tumors. *Cancer Res.* (1994) 54:6353-6358.
92. WASHIMI O, NAGATAKE M, OSADA H, UEDA R, KOSHIKAWA T, SEKI T, TAKAHASHI T, TAKAHASHI T: *In vivo* occurrence of *p16 (MTS1)* and *p15 (MTS2)* alterations preferentially in non-small cell lung cancers. *Cancer Res.* (1995) 55:514-517.
93. HUSSUSSIAN CJ, STRLEWING JP, GOLDSTEIN AM, HIGGINS PAT, ALLY DS, SHEAHAN MD, CLARK WH JR, TUCKER MA, DRACOPOLI NC: Germline *p16* mutation in familial melanoma. *Nature Genet.* (1994) 8:15-21.
94. CAI D W, MUKHOPADHYAY T, LIU Y, FUJIWARA T, ROTH JA: Stable expression of the wild-type *p53* gene in human lung cancer cells after retrovirus-mediated gene transfer. *Human Gene Ther.* (1993) 4:617-624.
95. FUJIWARA T, GRIMM EA, MUKHOPADHYAY T, CAI DW, OWEN-SCHAUB LB, ROTH JA: A retroviral wild-type *p53* expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res.* (1993) 53:4129-4133.
96. CASSON AG, MUKHOPADHYAY T, CLEARY KR, RO JY, LEVINE B, ROTH JA: *p53* mutations in Barrett's epithelium and esophageal cancer. *Cancer Res.* (1991) 51:4495-4499.
97. CHUNG KY, MUKHOPADHYAY T, KIM J, CASSON A, RO JY, GOEPFERT H, HONG WK, ROTH JA: Discordant *p53* gene mutations in primary head and neck cancers and corresponding second primary cancers of the aerodigestive tract. *Cancer Res.* (1993) 53:1676-1683.
98. ZHANG W-W, FANG X, BRANCH CD, MAZUR W, FRENCH BA, ROTH JA: Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. *BioTechniques* (1993) 15:868-872.  
First report of generation of recombinant *p53* adenovirus.
99. ZHANG W-W, FANG X, MAZUR W, FRENCH BA, GEORGES RN, ROTH JA: High-efficiency gene transfer and high-level expression of wild-type *p53* in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther.* (1994) 1:5-13.  
A significant report of the recombinant *p53* adenovirus and its application in lung cancer suppression.
100. WANG J, BUCANA CD, ROTH JA, ZHANG W-W: Apoptosis induced in human osteosarcoma cells is one of the mechanisms for the cytotoxic effect of Ad5CMV-*p53*. *Cancer Gene Ther.* (1995) 1:9-17.
101. WILLS KN, MANEVAL DC, MENZEL P, HARRIS MP, SUTJIPTO S, VAILLANCOURT M-T, HUANG W-M, JOHNSON DE, ANDERSON SC, WEN SF, BOOKSTEIN R, SHEPARD HM, GREGORY RJ: Development and characterization of recombinant adenoviruses encoding human *p53* for gene therapy of cancer. *Human Gene Ther.* (1994) 5:1079-1088.
102. LIU TJ, ZHANG W-W, TAYLOR DL, ROTH JA, GOEPFERT H, CLAYMAN GL: Growth suppression of human head and neck cancer cells by the introduction of a wild-type *p53* gene via a recombinant adenovirus. *Cancer Res.* (1994) 54:3662-3667.
103. MOOLTEN FL: Drug sensitivity ('suicide') genes for selective cancer chemotherapy. *Cancer Gene Ther.* (1994) 1:279-287.
104. MAXWELL IH, MAXWELL F, GLODE LM: Regulated expression of a diphtheria toxin A-chain gene transfected into human cells: possible strategy for inducing cancer cell suicide. *Cancer Res.* (1986) 46:4660-4664.
105. BREITMAN M L, CLAPOFF S, ROSSANT J, TSUI L C, GLODE LM, MAXWELL IH, BERNSTEIN A: Genetic ablation: programmed lineage suicide by tissue-specific expression of the diphtheria toxin A gene in transgenic mice. *Science* (1987) 238:1563-1565.  
First report of tissue-specific expression of a toxin gene *in vivo*.
106. PALMITER RD, BEHRINGER RR, QUAIFE CJ, MAXWELL F, MAXWELL IH, BRINSTER RL: Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell* (1987) 50:435-443.
107. YAMAIZUMI M, MEKADA E, UCHIDA T, OKADA Y: One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* (1978) 15:245-250.
108. MAXWELL IH, GLODE LM, MAXWELL F: Expression of diphtheria toxin A-chain in mature B-cells: a potential approach to therapy of B-lymphoid malignancy. *Leuk. Lymphoma* (1992) 7:457-462.
109. PURI RK, OGATA M, IELAND P, FELDMAN GM, FITZGERALD D, PASTAN I: Expression of high-affinity interleukin 4 receptors on murine sarcoma cells and receptor-mediated cytotoxicity of tumour cells to chimeric protein between interleukin 4 and *Pseudomonas* exotoxin. *Cancer Res.* (1991) 51:3011-3017.
110. RAO L, DEBBAS M, SABBATINI P, HOCKENBERRY D, KORSMEYER S, WHITE E: The adenovirus E1A proteins induce apoptosis which is inhibited by the E1B 19K and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA* (1992) 89:7742-7746.
111. DEBBAS M, WHITE E: Wild-type *p53* mediates apoptosis by E1A which is inhibited by E1B. *Gene Dev.* (1993) 7:546-554.
112. YU D, WOLF JK, SCANLON M, PRICE JE, HUNG MC: Enhanced *c-erbB-2/neu* expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Canc. Res.* (1993) 53:891-898.
113. ZHANG Y, YU D, XIA W, HUNG MC: *HER-2/neu*-targeting cancer therapy via adenovirus-mediated E1A delivery in an animal model. *Oncogene* (1995). In press.
114. ELION GB, FURMAN PA, FYFE JA, DE MIRANDA P, BEAUCHAMP L, SCHAEFFER HJ: Selectivity of action of an antihypertensive agent, 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci. USA* (1977) 74:5716-5720.
115. DAVIDSON RL, KAUFMAN ER, CRUMPACKER CS, SCHNITPER IE: Inhibition of herpes simplex virus transformed and nontransformed cells by acycloguanosine: mechanisms of uptake and toxicity. *Virology* (1981) 113:9-19.  
A mechanism study of acycloguanosine as anti-HSV agent.
116. MOOLTEN FL, WELLS JM: Curability of tumours bearing herpes thymidine kinase genes transferred by retroviral vectors. *J. Natl. Cancer Inst.* (1990) 82:297-300.
117. CULVER KW, RAM Z, WALLBRIDGE S, ISHII H, OLDFIELD EH, BLAASE RM: Vector-producer cells for treatment of



- experimental brain tumours. *Science* (1992) 256:1550-1552.
- A significant report of treatment of brain tumour model with HSV-*tk* gene transfer with systemic administration of GCV.
118. BI WL, PARYSEK LM, WARNICK R, STAMBROOK PJ: *In vitro* evidence that metabolic cooperation is responsible for the bystander effect observed with HSV-*tk* retroviral gene therapy. *Human Gene Ther.* (1993) 4:725-731.
  119. OLDFIELD EH: Gene therapy for the treatment of brain tumours using intratumoral transduction with the thymidine kinase gene and intravenous ganciclovir. *Human Gene Ther.* (1993) 4:36-69.
  120. HUBER BE, RICHARDS CA, KRENITSKY TA: Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy. *Proc. Natl. Acad. Sci. USA* (1991) 88:8039-8043.
- A significant report of design of tissue-specific expression of a prodrug-activation gene for gene therapy of cancer.
121. MULLEN CA, KILSTRUP M, BLAISE RM: Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc. Natl. Acad. Sci. USA* (1992) 89:33-37.
  122. GARVER RI, Jr., GOLDSMITH KT, RODU B, HU PC, SORSCHER EJ, CUREL DT: Strategy for achieving selective killing of carcinomas. *Gene Ther.* (1994) 1:46-50.
  123. MANOME Y, ABE M, HAGEN MF, FINE HA, KULFE DW: Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer cells to ganciclovir. *Cancer Res.* (1994) 54:5408-5413.
  124. SMITH MJ, ROUSCULP MD, GOLDSMITH KT, CUREL DT, GARVER RI, Jr.: Surfactant protein A-directed toxin gene kills lung cancer cells *in vivo*. *Human Gene Ther.* (1994) 5:29-35.
  125. DIMAJO JM, CLARY BM, VIA DF, COVENEY E, PAPPAS TN, LYERLY HK: Directed enzyme pro-drug gene therapy for pancreatic cancer *in vivo*. *Surgery* (1994) 116:205-213.
  126. WEI M-X, TAMIYA T, CHASE M, BOVIATIS EJ, CHANG TKH, KOWALL NW, HOCHBERG FH, WAXMAN DJ, BREAKFIELD XO, CHIOCCA EA: Experimental tumor therapy in mice using the cyclophosphamide-activating cytochrome P450 2B1 gene. *Human Gene Ther.* (1994) 5:969-978.
  127. WEICHELBAUM RR, HALLAHAN DE, BECKETT MA, MAUCERI HJ, LEE H, SUKHATME VP, KULFE DW: Gene therapy targeted by radiation preferentially radiosensitizes tumour cells. *Cancer Res.* (1994) 54:4266-4269.
  128. GOTTESMAN MM, GERMANN UA, AKSENTJEVICH I, SUGIMOTO Y, CARDARELLI CO, PASTAN I: Gene therapy of drug resistance genes: Implications for cancer therapy. *Annals New York Acad. Sci.* (1994) 716:126-138.
- A recent review of MDR genes and their application in cancer gene therapy.
129. PASTAN I, GOTTESMAN MM: Multiple-drug resistance in human cancer. *New Engl. J. Med.* (1987) 316:1388-1393.
  130. SHEN DW, CARDARELLI C, HWANG J, CORNWELL MM, RICHERT N, ISHII SI, PASTAN I, GOTTESMAN MM: Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. *J. Biol. Chem.* (1986) 261:7762-7770.
  131. RONINSON IB, CHIN JE, CHOI K, GROS P, HOUSMAN DE, FOJO A, SHEN DW, GOTTESMAN MM, PASTAN I: Isolation of the human *mdr* DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc. Natl. Acad. Sci. USA* (1986) 83:4538-4542.
- A significant report of isolation of the human *MDR1* gene.
132. CHEN CJ, CHIN JE, UEDA K, CLARK D, PASTAN I, GOTTESMAN MM, RONINSON IB: Internal duplication and homology with bacterial transport protein in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* (1986) 47:381-389.
  133. JURANA PF, ZASTAWNY RL, LING V: P-glycoprotein: Multidrug-resistance and a superfamily of membrane-associated transport proteins. *FASEB J.* (1989) 3:2583-2592.
- A detailed review on multidrug-resistance and a superfamily of membrane-associated transport proteins.
134. GOTTESMAN MM, PASTAN I: Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann. Rev. Biochem.* (1993) 62:385-427.
  135. RIORDAN JR, DEUCHARS K, KARTNER N, ALON N, TRENT J, LING V: Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* (1985) 316:817-819.
  136. GROS P, NERIAH YB, CROOP JM, HOUSMAN DE: Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* (1986) 323:728-731.
  137. SHEN DW, FOJO A, RONINSON IB, CHIN JE, SOFFIR P, PASTAN I, GOTTESMAN MM: Multidrug resistance of DNA-mediated transformants is linked to transfer of the human *mdr1* gene. *Mol. Cell. Biol.* (1986) 6:4039-4044.
  138. GALSKE H, SULLIVAN M, WILLINGHAM MC, CHIN KV, GOTTESMAN MM, PASTAN I, MERLINO GT: Expression of a human multidrug resistance cDNA (*MDR1*) in the bone marrow of transgenic mice: Resistance to daunomycin-induced leukopenia. *Mol. Cell. Biol.* (1989) 9:4357-4363.
- A study of the *MDR1* transgenic mouse model.
139. MORROW CS, COWAN KH: Mechanisms and clinical significance of multidrug resistance. *Oncology* (1988) 2:55-67.
  140. BECK WT: Mechanisms of multidrug resistance in human tumour cells. The roles of P-glycoprotein, DNA topoisomerase II, and other factors. *Cancer Treat. Rev.* (1990) 17(Suppl. A):11-20.
  141. FINE RL, PATEL J, CHABNER BA: Phorbol esters induce multidrug resistance in human breast cancer cells. *Proc. Natl. Acad. Sci. USA* (1988) 85:582-586.
  142. TAYLOR CW, BRATTAIN MG, YEOMAN LC: Occurrence of cytosolic protein and phosphoprotein changes in human colon tumor cells with the development of resistance to mitomycin C. *Cancer Res.* (1985) 45:4422-4427.
  143. WANG AL, TEW KD: Increased glutathione-S-transferase activity in a cell line with acquired resistance to nitrogen mustards. *Cancer Treat. Rep.* (1985) 69:677-682.
  144. MCGOWN AT, FOX BW: A proposed mechanism of resistance to cyclophosphamide and phosphoramidate mustard in a Yoshida cell line *in vitro*. *Cancer Chemother. Pharmacol.* (1986) 17:223-227.

145. ROBSON CN, LEWIS AD, WOLF CR, HAYES JD, HALL A, PROCTOR SJ, HARRIS AL, HICKSON ID: Reduced levels of drug-induced DNA cross-linking in nitrogen mustard-resistant Chinese hamster ovary cells expressing elevated glutathione-S-transferase activity. *Cancer Res.* (1987) 47:6022-6027.
146. MORROW CS, COWAN KH: Multidrug resistance associated with altered topoisomerase II activity-topoisomerase II as targets for rational drug design. *J. Natl. Cancer Inst.* (1990) 82:638-639.
147. DEFFIE AM, BATRA JK, GOLDENBERG JG: Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukemia cell lines. *Cancer Res.* (1989) 49:58-62.
148. SAVAS B, COLE SPC, AKOGLU TF, PROSS HF: P-glycoprotein-mediated multidrug resistance and cytotoxic effector cells. *Nat. Immun.* (1992) 11:177-192.
149. MICKISCH GH, AKSENTJEVICH I, SCHOENLEIN PV, GOLDSTEIN IJ, GALSKE H, STAHL C, SACHS DH, PASTAN I, GOTTESMAN MM: Transplantation of bone marrow cells from transgenic mice expressing the human MDR1 gene results in long-term protection against the myelosuppressive effect of chemotherapy in mice. *Blood* (1992) 79:1-7.
150. PODDA S, WARD M, HIMELSTEIN A, RICHARDSON C, FLOR-WEISS E, SMITH L, GOTTESMAN MM, PASTAN I, BANK A: Transfer and expression of the human multiple drug resistance (MDR) gene into live mice. *Proc. Natl. Acad. Sci. USA* (1992) 89:9676-9680.
151. SORRENTINO BP, BRANDT SJ, BODINE D, GOTTESMAN MM, PASTAN I, CLINE A, NIENHUIS AW: Retroviral transfer of the human MDR1 gene permits selection of drug-resistant bone marrow cells *in vivo*. *Science* (1992) 257:99-103.  
A significant report of *in vivo* selection of drug-resistant bone marrow cells after transfer with the MDR1 gene: implication for chemoprevention.
152. HANANIA EG, FU SQ, RONINSON I, ZU Z, GOTTESMAN MM, HEGEWISCH-BECKER S, ANDREEFF M, DEISSEROTH AB: Resistance to Taxol chemotherapy engineered by safety-modified retroviruses containing an MDR-1 transcription unit. *Gene Ther.* (1995). In press.
153. HANANIA EG, DEISSEROTH AB: Serial transplantation shows that early hematopoietic precursor cells are transduced by MDR-1 retroviral vector in a mouse gene therapy model. *Cancer Gene Ther.* (1994) 1:21-25.
154. COREY CA, DESILVA AD, HOLLAND CA, WILLIAMS DA: Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells. *Blood* (1990) 75:337-343.
155. SURESH A, TUNG E, MOREB J, JAMES RZ: Role of manganese superoxide dismutase in radioprotection using gene transfer studies. *Cancer Gene Ther.* (1994) 1:85-90.
156. ROSENBERG SA, AEBERSOLD P, CORNETTA K, KASID A, MORGAN RA, MOEN R, KARSON EM, LOTZE MT, YANG JC, TOPALIAN SL, MERINO MJ, CULVER K, MILLER AD, BLAESE RM, ANDERSON WF: Gene transfer into humans: immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *New Engl. J. Med.* (1990) 323:570-578.  
First report of *ex vivo* gene transfer into human.
157. BLAESE RM, ANDERSON WF: The ADA human gene therapy clinical protocol. *Human Gene Ther.* (1990) 1:327-329.
158. ROSENBERG SA, SPIESS P, LAFRENIERE R: A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* (1986) 233:1318-1321.  
An introduction about application of TIL for cancer therapy.
159. MULL LM, SPIESS PJ, DIRECTOR EP, ROSENBERG SA: Identification of specific cytolytic immune responses against autologous tumor in humans bearing malignant melanoma. *J. Immunol.* (1987) 138:989-995.
160. BARTH RJ, BOCK SN, MULE JJ, ROSENBERG SA: Unique murine tumor associated antigens identified by tumor-infiltrating lymphocytes. *J. Immunol.* (1990). 144:1531-1537.
161. BARTH RJ, MULE JJ, SPIESS PJ, ROSENBERG SA: Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8<sup>+</sup> tumor-infiltrating lymphocytes. *J. Exp. Med.* (1991). 173:647-658.
162. TOPALIAN SL, SOLOMON D, ROSENBERG SA: Tumor-specific cytotoxicity by lymphocytes infiltrating human melanoma. *J. Immunol.* (1989) 142:3714-3725.
163. FISHER B, PAKARD BS, READ EJ, CARRASQUILLO JA, CARTER CS, TOPALIAN S, YANG JC, YOLLES P, LARSON SM, ROSENBERG SA: Tumor localization of adoptively transferred Indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma. *J. Clin. Oncol.* (1989) 7:250-261.
164. GRIFFITH KD, READ EJ, CARRASQUILLO JA, CARTER CS, YANG JC, FISHER B, AEBERSOLD P, PACKARD BS, YU MY, ROSENBERG SA: *In vivo* distribution of adoptively transferred Indium-111 labeled tumor-infiltrating lymphocytes and peripheral blood lymphocytes in patients with metastatic melanoma. *J. Natl. Cancer Inst.* (1989) 81:1709-1717.
165. ROSENBERG SA, PACKARD BS, AEBERSOLD PM, SOLOMON D, TOPALIAN SL, TOY ST, SIMON P, LOTZ MT, YANG JC, SEIPP CA, SIMPSON C, CARTER C, BOOK S, SCHWARTZENTRUBER D, WEI JP, WHITE DE: Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: a preliminary report. *New Engl. J. Med.* (1988) 319:1676-1680.
166. KASID A, MORECKI S, AEBERSOLD P, CORNETTA K, CULVER K, FREEMAN S, DIRECTOR E, LOTZ MT, BLAESE RM, ANDERSON WF, ROSENBERG SA: Human gene transfer: characterization of human tumor-infiltrating lymphocytes as vehicles for retroviral-mediated gene transfer in man. *Proc. Natl. Acad. Sci. USA* (1990) 87:473-477.
167. RILL DR, MOEN RC, BUSCHLE M, BARTHOLOMEW C, FOREMAN NK, MIRRO J, KRANCE RA, IHLE JN, BRENNER MK: An approach for the analysis of relapse and marrow reconstitution after autologous marrow transplantation using retrovirus-mediated gene transfer. *Blood* (1992) 79:2694-2700.
168. DEISSEROTH AB: Use of two retroviral markers to test relative contribution of marrow and peripheral blood autologous cells to recovery after preparative therapy. *Human Gene Ther.* (1993) 4:71-85.
169. ASHER AL, MULE JJ, REICHERT CM, SHILONI E, ROSENBERG SA: Studies on the anti-tumor efficacy of systemically administered recombinant tumor necrosis factor



- against several murine tumors *in vivo*. *J. Immunol.* (1987) 138:963-974.
170. ROSENBERG SA: Immunization of cancer patients using autologous cancer cells modified by insertion of the gene for tumor necrosis factor. *Human Gene Ther.* (1992) 3:57-73.
  171. ROSENBERG SA: Gene therapy for cancer. In: *Important Advances in Oncology*. DeVita VT, Jr., Hellman S, Rosenberg SA (Eds.). JB Lippincott Company, Philadelphia (1992):17-38.
  172. HWU P, YANNELLI J, KRIEGLER M, ANDERSON WF, PEREZ C, CHIANG Y, SCHEARZ S, COWHERD R, DELGADO C, MULE J, ROSENBERG SA: Functional and molecular characterization of tumor-infiltrating lymphocytes transduced with tumor necrosis factor- $\alpha$  cDNA for the gene therapy of cancer in humans. *J. Immunol.* (1993) 150:4104-4115.
  173. ESHHAR Z: Redirection of effector lymphocytes to tumor cells using chimeric receptors with antibody specificity. *Cancer Gene Ther.* (1994) 1:139.
  174. ESHHAR Z, WAKS T, GROSS G, SCHINDLER D: Specific activation and targeting of cytotoxic lymphocytes through chimeric single-chains consisting of antibody-binding domains and the  $\gamma$  or  $\zeta$  subunits of the immunoglobulin and T-cell receptors. *Proc. Natl. Acad. Sci. USA* (1993) 90:720-724.
  175. MORITZ D, WELS W, MATTERN J, GRONER B: Cytotoxic T-lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc. Natl. Acad. Sci. USA* (1994) 91:4318-4322.
  176. OETTGEN HF, OLD LJ: The history of cancer immunotherapy. In: *Biologic Therapy of Cancer*. DeVita VT, Hellman S, Rosenberg SA (Eds.). JB Lippincott, Philadelphia (1991):87-119.
  177. VAAGE J: Peritumor interleukin-2 causes systemic therapeutic effect via interferon- $\gamma$  induction. *Int. J. Cancer* (1991) 49:598-600.
  178. HULAND E, HULAND H, HEINZER H: Interleukin-2 by inhalation: local therapy for metastatic renal cell carcinoma. *J. Urol.* (1992) 147:344-348.
  179. COLOMBO M, MODESTI A, PARMIANI G, FORNI G: Local cytokine availability elicits tumor rejection and systemic immunity through granulocyte-T-lymphocyte cross-talk. *Cancer Res.* (1992) 52:4853-4857.
  180. TEPPER RI, MULE JJ: Experimental and clinical studies of cytokine gene-modified tumor cells. *Human Gene Ther.* (1994) 5:153-164.
- A detailed review on cytokine gene-modified tumor cells as tumor vaccines.
181. LOTZE MT: Role of IL-4 in the antitumor response. In: *IL-4: Structure and Function*. Spits H (Ed.). CRC Press, Boca Raton, FL (1991) 147:2950-2956.
  182. TEPPER RI, PATTENGAL PK, LEDER P: Murine interleukin-4 displays potent antitumor activity *in vivo*. *Cell* (1989) 57:503-512.
  183. GOLUMBEK PT, LAZENBY AJ, LEVITSKY HI, JAFFEE LM, KARASUYAMA H, BAKER M, PARDOLL DM: Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science* (1991) 254:713-716.
- An early study of the cytokine gene-modified tumour vaccine.
184. PIPPIN BA, ROSENSTEIN M, JACOB WF, CHIANG Y, LOTZE MT: Local IL-4 delivery enhances immune reactivity to murine tumors: Gene therapy in combination with IL-2. *Cancer Gene Ther.* (1994) 1:35-42.
  185. SMITH KA: Interleukin-2: Inception, impact, and implications. *Science* (1988) 240:1169-1176.
- A detailed review of IL-2.
186. GRIMM EA, MAZUMDER A, ZHANG HZ, ROSENBERG SA: Lymphokine-activated killer phenomenon. Lysis of natural killer resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.* (1982) 155:1823-1841.
  187. FEARON E, PARDOLL D, ITAYA T, GOLUMBEK P, LEVITSKY H, SIMONS J, KARASUYAMA H, VOGELSTEIN B, FROST P: Interleukin-2 production by tumor cells bypasses T-helper function in the generation of an antitumor response. *Cell* (1990) 60:397-403.
  188. GANSBACHER B, ZIER K, DANIELS B, CRONIN K, BANNERJI R, GILBOA E: Interleukin-2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J. Exp. Med.* (1990) 172:1217-1224.
  189. BUBENIK J, SIMOVA J, JANDLOVA T: Immunotherapy of cancer using local administration of lymphoid cells expressing IL-2 cDNA and constitutively producing IL-2. *Immunol. Lett.* (1990) 23:287-292.
  190. PORGADOR A, GANSBACHER B, BANNERJI R, TZEHOVAL E, GILBOA E, FELDMAN M, EISENBACH I: Anti-metastatic vaccination of tumor-bearing mice with IL-2-gene-inserted tumor cells. *Int. J. Cancer* (1993) 53:471-477.
  191. TSAI SCJ, GANSBACHER B, TAIT L, MILLER FR, HEPPNER GH: Induction of antitumor immunity by interleukin-2 gene-transduced mouse mammary tumor cells versus transduced mammary stromal fibroblasts. *J. Natl. Cancer Inst.* (1993) 85:546-553.
  192. GANSBACHER B, ZIER K, CRONIN K, HANTZOPOULOS PA, BOUCHARD B, HOUGHTON A, GILBOA E, GOLDE D: Retroviral gene transfer induced constitutive expression of interleukin-2 or interferon- $\gamma$  in irradiated human melanoma cells. *Blood* (1992) 80:2817-2825.
  193. HADDADA H, RAGOT T, CORDIER L, DUFFOUR MT, PERICADUET M: Adenoviral interleukin-2 gene transfer into p815 tumor cells abrogates tumorigenicity and induces antitumoral immunity in mice. *Human Gene Ther.* (1993) 4:703-711.
  194. GASTL G, FINSTAD CL, GUARINI A, BOSL G, GILBOA E, BANDER NH, GANSBACHER B: Retroviral vector-mediated lymphokine gene transfer into human renal cancer cells. *Cancer Res.* (1992) 52:6229-6236.
  195. ZIER KS, SALVADORI S, CRONIN KC, GANSBACHER B: Vaccination with IL-2-secreting tumor cells stimulates the generation of IL-2-responsive T-cells and prevents the development of unresponsiveness. *Cancer Gene Ther.* (1994) 1:43-50.
  196. FOA R, GUARINI A, GANSBACHER B: IL-2 treatment for cancer: from biology to gene therapy. *Br. J. Cancer* (1992) 66:992-998.
- A detailed review on IL-2 and its application for tumour vaccination.
197. DALGLEISH AG: The role of IL-2 in gene therapy. *Gene Ther.* (1994) 1:83-87.
  198. WALLACH D, FELLOUS M, REVEL M: Preferential effect of interferon- $\gamma$  on the synthesis of HLA-antigens and their mRNAs in human cells. *Nature* (1982) 299:833-836.

199. CHEN LK, TOURVIEILLE B, BURNS GF, BACH FH, MATIEU-MAHUL D, SASPORTES M, BENSUSSAN A: Interferon- $\gamma$  a cytotoxic T-lymphocyte differentiation signal. *Eur. J. Immunol.* (1986) 16:767-770.
  200. NICHOLAS PR, SPIESS PJ, KARP SE, MULE JJ, ROSENBERG SA: A nonimmunogenic sarcoma transduced with cDNA for interferon- $\gamma$  elicits CD8<sup>+</sup> T-cells against the wild-type tumor: correlation with antigen presentation capability. *J. Exp. Med.* (1992) 175:1423-1431.
  201. PORGADOR A, BANNERJI R, WATANABE Y, FELDMAN M, GILBOA E, EISENBACH L: Antimetastatic vaccination of tumor-bearing mice with two types of IFN- $\gamma$  gene-inserted tumor cells. *J. Immunol.* (1993) 150:1458-1470.
  202. ABDEL-WAHAB ZA, OSANTO S, DARROW TL, BARBER JR, VERVAERT CE, GANGAVALLI R, MCCALLISTER TJ, SEIGLER HF: Transduction of human melanoma cells with the gamma interferon gene enhances cellular immunity. *Cancer Gene Ther.* (1994) 1:171-179.
  203. COLLINS T, LAPIERRE LA, FIERIS W, STROMINGER JL, POBER JS: Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A, B antigens in vascular endothelial cells and dermal fibroblasts *in vitro*. *Proc. Natl. Acad. Sci. USA* (1986) 83:446-450.
  204. CARSWELL EA, OLD LJ, KASSEL RL, GREEN S, FIORE N, WILLIAMSON B: An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA* (1975) 72:3666-3670.
  205. HARANAKA K, SATOMI N, SAKURAI A: Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice. *Int. J. Cancer* (1984) 34:263-267.
  206. SIDHU RS, BOLLON AP: Tumor necrosis factor activities and cancer therapy - a perspective. *Pharmacol. Ther.* (1993) 57:79-128.
  207. ASHER AL, MULE JJ, KASID A, RESTIFO NP, SALO JC, REICHERT CM, JAFFE G, FENDLY B, KRIEGLER M, ROSENBERG SA: Murine tumor cells transduced with the gene for tumor necrosis factor- $\alpha$ : Evidence for paracrine immune effects of tumor necrosis factor against tumors. *J. Immunol.* (1991) 146:3227-3234.
  208. BLANKENSTEIN T, QIN Z, UBERLA K, MÜLLER W, ROSEN H, VOLK HD, DIAMANTSTEIN T: Tumor suppression after tumor cell-targeted tumor necrosis factor gene transfer. *J. Exp. Med.* (1991) 173:1047-1052.
  209. KARP SE, FARBER A, SALO JC, HWU P, JAFFE G, ASHER AL, SHILONI E, RESTIFO NR, MULE JJ, ROSENBERG SA: Cytokine secretion by genetically modified nonimmunogenic murine fibrosarcoma. *J. Immunol.* (1993) 150:896-908.
  210. STOTTER H, CUSTER MC, BOLTON ES, GUEDEZ L, LOTZE MT: IL-7 induces human lymphokine-activated killer cell activity and is regulated by IL-4. *J. Immunol.* (1991) 146:150-155.
  211. ALDERSON MR, TOUGH TW, ZIEGLER SF, GRABSTEIN KH: Interleukin-7 induces cytokine secretion and tumoricidal activity by human peripheral blood monocytes. *J. Exp. Med.* (1991) 173:923-930.
  212. HOCK H, DORSCH M, DIAMANTSTEIN T, BLANKENSTEIN T: Interleukin-7 induces CD4<sup>+</sup> T-cell-dependent tumor rejection. *J. Exp. Med.* (1991) 174:1291-1298.
  213. MCBRIDE WH, THACKER JD, COMORA S, ECONOMOU JS, KELLY D, HOGGE D, DUBINETT SM, DOUGHERTY GJ: Genetic modification of a murine fibrosarcoma to produce interleukin-7 stimulates host cell infiltration and tumor immunity. *Cancer Res.* (1992) 52:3931-3937.
  214. JICHA DL, MULE JJ, ROSENBERG SA: Interleukin-7 generates antitumor CTL against murine sarcomas with efficacy in cellular adoptive immunotherapy. *J. Exp. Med.* (1991) 174:1511-1515.
  215. STEINMAN RM: The dendritic cell system and its role in immunogenicity. *Ann. Rev. Immunol.* (1991) 9:271-296.
  216. INABA K, INABA MN, ROMANI N, AYA H, STEINMAN S: Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* (1992) 176:1693-1703.
  217. DRANOFF G, JAFFEE E, LAZENBY A, GOLUMBEK P, LEVITSKY H, BROSE K, JACKSON V, HAMADA H, PARDOLL D, MULLIGAN R: Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting antitumor immunity. *Proc. Natl. Acad. Sci. USA* (1993) 90:3539-3543.
- A significant report on the GM-CSF gene-modified tumour vaccination and comparison with the effects of other cytokine genes.
218. NICOLA NA, METCALF D, MATSUMOTO M, JOHNSON GR: Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells: identification as granulocyte colony-stimulating factor (G-CSF). *J. Biol. Chem.* (1983) 258:9017-9023.
  219. COLOMBO MP, FERRARI G, STOPPACCIARO A, PARENZA M, RODOLFO M, MAVILIO F, PARMIANI G: Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma *in vivo*. *J. Exp. Med.* (1991) 173:889-897.
  220. COLOMBO MP, LOMBARDI L, STOPPACCIARO A, MELANI C, PARENZA M, BOTTAZZI B, PARMIANI G: Granulocyte colony-stimulating factor (G-CSF) gene transduction in murine adenocarcinoma drives neutrophil-mediated tumor inhibition *in vivo*: neutrophils discriminate between G-CSF-producing and G-CSF-nonproducing tumor cells. *J. Immunol.* (1992) 149:113-119.
  221. STOPPACCIARO A, MELANI C, PARENZA M, MASTRACCHIO A, BASSI C, BARONI C, PARMIANI G, COLOMBO MP: Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T-cell-produced interferon- $\gamma$ . *J. Exp. Med.* (1993) 178:151-161.
  222. OSTRAND-ROSENBERG S, THAKUR A, CLEMENTS V: Rejection of mouse sarcoma cells after transfection of MHC class II gene. *J. Immunol.* (1990) 144:4068-4071.
  223. SCHULTZ Z, KLARNET J, GIENI R, HAYGLASS K, GREENBERG P: The role of B-cells for *in vitro* T-cell responses to a Friend virus-induced leukemia. *Science* (1990) 249:921-923.
  224. GREENBERG PD: Adoptive T-cell therapy of tumors: Mechanisms operative in the recognition and elimination of tumor cells. *Adv. Immunol.* (1990) 49:281-355.
  225. MUELLER D, JENKINS M, SCHWARTZ R: Clonal expansion versus function clonal inactivation: a co-stimulatory signalling pathway determines the outcome of T-cell antigen receptor occupancy. *Ann. Rev. Immunol.* (1989) 7:445-480.
  226. GIMMI CD, FREEMAN GJ, GRIBBEN JG, SUGITA K, FREEDMAN AS, MORIMOTO C, NADLER LM: B-cell surface anti-



# RECOMBINANT ADENOVIRUS VECTOR EXPRESSING WILD-TYPE p53 IS A POTENT INHIBITOR OF PROSTATE CANCER CELL PROLIFERATION\*

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**ABSTRACT—Objectives.** A recombinant adenovirus vector (AdWtp53) expressing wild-type p53 was evaluated for its cell growth inhibitory effects on metastatic human prostate cancer cells.

**Methods.** Human prostate cancer cells LNCaP, DU145, PC3, 1LN, and DUPro-1 were infected with AdWtp53 vector and expression of exogenous p53 in these cells was analyzed by immunoprecipitation and western blot assays. The cell growth inhibitory effects of AdWtp53 were determined by counting cell number on a hemocytometer or by crystal violet staining of cells after infection with AdWtp53. The p53-regulated gene WAF1 and DNA fragmentation were also analyzed in prostate cancer cells infected with AdWtp53.

**Results.** High levels of the AdWtp53 vector-derived p53 protein were present in metastatic prostate cancer cells, and the p53-regulated gene WAF1 was induced in these cells. Infection of these tumor cell lines with AdWtp53 vector resulted in severe growth inhibition and cell death in comparison to untreated or control adenovirus vector-infected cells. Furthermore, fragmentation of genomic DNA, a property associated with apoptosis, was also observed in prostate cancer cells infected with AdWtp53.

**Conclusions.** AdWtp53 vector exhibited a potent inhibitory effect on the growth of all of human metastatic prostate cancer cells, and both cytostatic and cytotoxic effects of AdWtp53 were observed. The induction of p53-regulated gene WAF1 in AdWtp53-infected prostate cancer cells suggests the involvement of cellular p53 pathway in the cell growth inhibition. These results provide a molecular basis for further evaluation of antitumor effects of AdWtp53 vector in animal models of prostate cancer. *UROLOGY* 46: 843–848, 1995.

The tumor suppressor gene p53 is one of the most frequently altered genes detected in human tumors. Both somatic and germline mutations of p53 have been reported.<sup>1,2</sup> Numerous early studies of p53 alterations in prostate cancers have revealed a low incidence (10% to 20% of specimens analyzed) of p53 mutations in prostate cancers.<sup>3,4</sup> However, more recent studies suggest that

p53 alterations may be frequent in a subset of prostate cancers, especially in hormone refractory disease.<sup>5-9</sup> The tumor suppressor activity of the p53 gene has been demonstrated for diverse tumor cell types,<sup>1</sup> including prostate tumor cells.<sup>10</sup> The biochemical/biologic functions of the native p53 protein include target gene transactivation, cell cycle checkpoint control, and programmed cell death.<sup>1,11,12</sup> One or more of these functions are known to be deregulated in tumor cells.<sup>12</sup> Thus, correction of p53 dysfunctions in human cancers may have widespread application in anticancer therapy. Adenovirus vectors containing potential therapeutic genes are currently being evaluated for their utility in gene therapy of cancer and other human diseases.<sup>13</sup> Adenovirus-based vectors are internalized into epithelial cells with an efficient receptor-mediated endocytosis, can be grown to high titers, and express a transgene to high levels without integrating into the cellular genome.<sup>13-15</sup> Recent reports of antiproliferative and antitumor effects of a recombinant adenovirus vector expressing wild-type (wt) p53 in nonsmall cell lung cancer cells<sup>16</sup> and head and neck cancer cells<sup>17</sup> in vitro and in nude mice provide early insights

*The opinions and assertions contained herein are the private views of the authors and should not be construed as reflecting the views of the Department of Defense or the US Army.*

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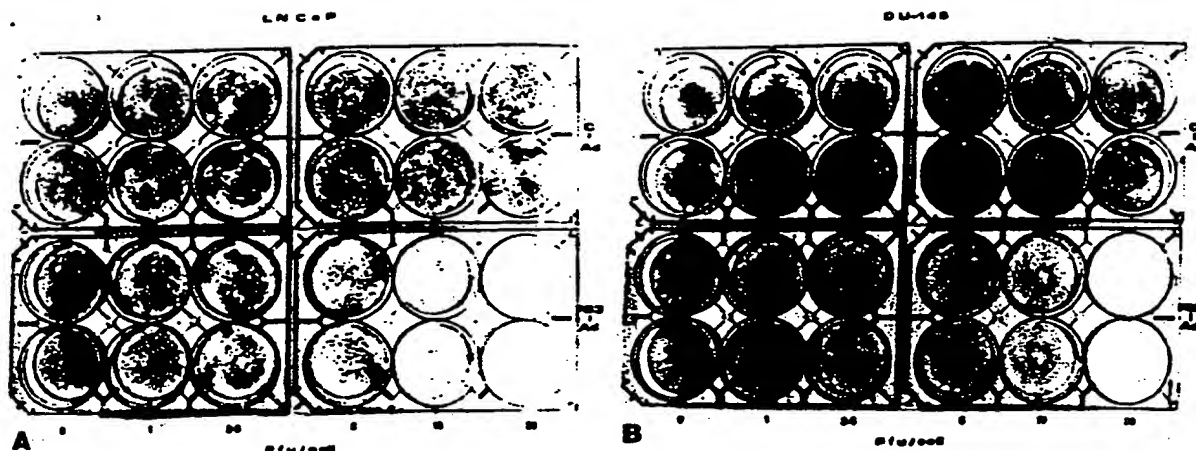


FIGURE 2. AdWTP53-mediated inhibition of prostate cancer cell proliferation. LNCaP (A) and DU145 (B) cells were treated with different dosages (plaque-forming unit/cell) of control adenovirus or AdWTP53 shown here as C-Ad or p53-Ad, respectively. The cells were fixed and stained with crystal violet after 96 hours of the infection. Violet stain shown in black here represents the presence of cells attached to tissue culture dish.

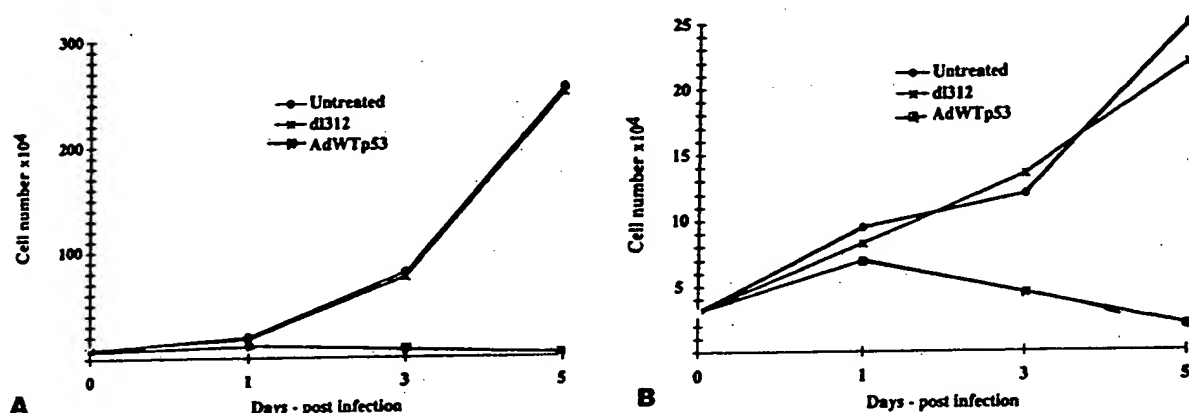


FIGURE 3. Time course of AdWTP53-mediated inhibition of prostate cancer cell growth. DU145 (A) and LNCaP (B) cells infected with control adenovirus: dl 312 or AdWTP53 vector were analyzed for their growth at different times after infection. Uninfected cells were also analyzed in parallel. The cell growth was monitored by counting cells on the hemocytometer at time 0 and at indicated times postinfection.

gels to x-ray film revealed faint endogenous p53 bands in C-Ad-infected DU145 and LNCaP cells (data not shown).

#### GROWTH INHIBITORY EFFECTS OF ADWTP53 ON PROSTATE CANCER CELLS

To analyze for effects of AdWTP53 on growth of prostate cancer cells, we infected DU145 and LNCaP cells with varying dosage of AdWTP53 or a control adenovirus vector. AdWTP53-infected cells began to exhibit growth inhibition at 2.5 plaque forming unit (pfu)/cell with a marked growth inhibition (more than 90%) between 10 to 20 pfu/cell (Fig. 2A, B). Similar results were also obtained from two other metastatic prostate cancer cells: 1LN and DUPro-1 (data not shown). Although there was some inhibitory effect (10% to 15%) on cell growth at or above 20 pfu/cell of C-Ad, several independent experiments showed a

dramatic growth inhibition (95% to 99%) of prostate cancer cells after 5 to 6 days in the presence of 10 to 20 pfu/cell of AdWTP53. We also did not observe any cytotoxic or growth inhibitory effects of AdWTP53 on human fibroblast cells. Although level of p53 in AdWTP53-infected H500 was not as high as in prostate cancer cells, it was at least fivefold over endogenous levels (data not shown). Since several previous studies did not observe cell growth inhibitory effects of exogenous p53 in tumor cells that already contained endogenous wt p53,<sup>24,25</sup> the inhibitory effects of AdWTP53 on LNCaP cells containing endogenous wt p53 was unexpected. However, in agreement with the previous observations,<sup>18</sup> we also did not detect a growth inhibitory effect of AdWTP53 on breast cancer cells, MCF7 containing endogenous wt p53 (data not shown). The kinetics of cell growth inhibition in response to AdWTP53 were further evaluated for two representative cell

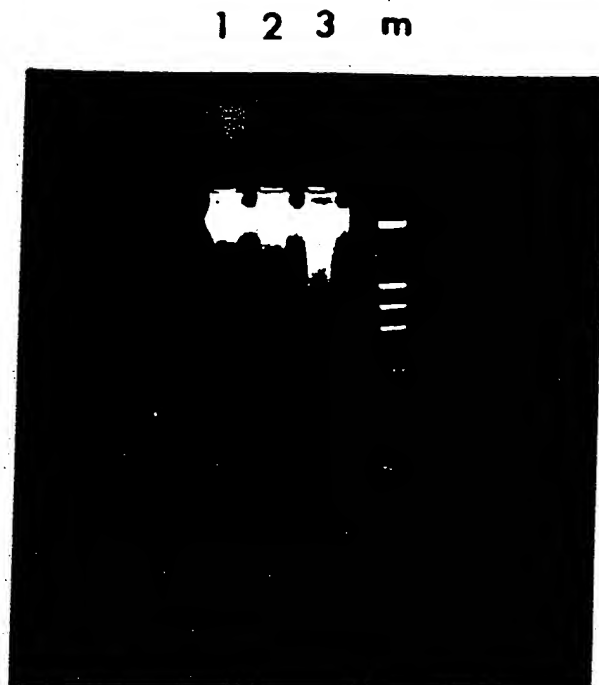


FIGURE 4. Analysis of genomic DNA from LNCaP cells infected with AdWTP53. Total genomic DNA was isolated from untreated LNCaP cells (lane 1), cells infected with 20 plaque-forming unit/cell of control adenovirus (lane 2), or AdWTP53 (lane 3) and analyzed on 2.5% agarose gel followed by ethidium bromide staining. m:  $\lambda$  HindIII + 1  $\times$  HaeIII DNA molecular weight marker.

lines: DU145 and LNCaP. As shown in Figure 3, DU145 cells exhibited almost complete inhibition by day 3, whereas LNCaP (data not shown) cells exhibited 95% inhibition by day 5. Furthermore, infection of  $1 \times 10^6$  cells of DU145 or LNCaP with

20 pfu/cell of AdWTP53 resulted in a large number of floating cells (representing 50% to 60% of total cells) within 48 to 50 hours, and more than 95% of these floating cells were nonviable as determined by trypan blue exclusion assay (data not shown). Additionally, we analyzed the genomic DNA from AdWTP53-infected LNCaP or DU145 cells for DNA fragmentation, a property known to be associated with programmed cell death. The analysis of genomic DNA from LNCaP (Fig. 4) and DU145 (data not shown) cells revealed DNA fragmentation in the AdWTP53-treated cells, whereas untreated or control adenovirus injected cells did not show any evidence of detectable DNA fragmentation.

#### INDUCTION OF WAF-1/CIP-1 BY AdWTP53 OR DOXORUBICIN IN PROSTATE CANCER CELLS

The expression of WAF1/CIP1 encoded p21 protein, an inhibitor of the cyclin-dependent kinases, has been shown to be directly up regulated by the wt p53.<sup>26</sup> Although WAF1 p21 protein was not readily detectable in PC3, LNCaP and DU145 cells (Fig. 5A) similar steady-state level of WAF1 RNA was detected in these cells (data not shown). All three prostate cancer cell lines, PC3, DU145, and LNCaP infected with AdWTP53, exhibited increased levels of WAF1/CIP1 p21 protein in comparison to the respective cell lines infected with C-Ad (Fig. 5A). To ascertain that LNCaP cells used in this study were not variant and did contain endogenous wt p53 function, we assayed the induction of WAF1 protein in LNCaP cells in response to doxorubicin, a chemotherapeutic agent reported to induce WAF1 only in those cells that contain wt p53.<sup>27</sup> As shown in Figure 5B, doxorubicin resulted in the induction of WAF1 p21



A



B

FIGURE 5. Analysis of WAF1 in prostate cancer cells. (A) WAF1/CIP1 induction in AdWTP53 infected cells: LNCaP (lanes 1 and 2), DU145 (lanes 3 and 4), and PC3 (lanes 5 and 6). Lanes 1, 3, and 5: cells infected with control adenovirus vector; lanes 2, 4, and 6: cells infected with AdWTP53 vector. Immunoprecipitation of WAF1 protein was performed as described under "Material and Methods." (B) Effect of doxorubicin treatment on the induction of WAF1/CIP1: prostate cancer cells ( $2 \times 10^6$ ); LNCaP (lanes 1, 2), PC3 (lanes 3, 4), DU145 (lanes 5, 6), and human fibroblast cells ( $2 \times 10^6$ ); H500 (lanes 7, 8) were treated with doxorubicin (lanes 2, 4, 6, and 8) or phosphate-buffered saline (lanes 1, 3, 5, and 7). Immunoblotting of WAF1 protein was performed on cell lysates and described under "Material and Methods."

protein in LNCaP cells (lane 2) and in normal human fibroblasts, H500 (lane 8). However, WAF1 p21 protein induction was not seen in PC3 (lane 4) or DU145 (lane 6) cells.

#### COMMENT

Gene therapeutic strategies for human prostate cancer encompass different approaches and represent an early phase of research in this direction. There are few reports that have described promising results with gene/immunotherapy of metastatic prostate cancer in an animal model system.<sup>28,29</sup> In these studies, genetically engineered prostate cancer cells producing recombinant cytokines provided effective therapy for prostate cancer in the Dunning rat prostate carcinoma model. The tumor vaccine approach described before is promising; however, the technology involved is very complicated and the major limitations precluding its wider application could be the availability and engineering of prostate cancer cells from the same patient in which it is to be utilized as vaccine. Other emerging molecular approaches for anti-cancer therapy include corrective gene therapy to correct a known molecular defect in cancer cells, for example, inhibition of an activated oncogene by antisense RNA/oligonucleotide or reconstitution of a defective or absent tumor suppressor gene (TSG) function by reintroduction of the normal copy of that TSG. The antiproliferative or antitumorogenic properties of the TSGs strongly suggest their therapeutic potential. However, the current challenges include the generation of efficient vectors for these potentially therapeutic genes and their delivery to cancer cells. Antiproliferative or antitumorogenic effects of the TSG p53 have been described for diverse types of tumor cells.<sup>1</sup> In this report, we have characterized the cell growth inhibitory effects of a recombinant adenovirus vector expressing high levels of wt p53 in human metastatic prostate cancer cells.

The expression of wt p53 is achieved at a very high level (at least 100-fold in comparison to the endogenous p53 bands seen in LNCaP or DU145 cells) in AdWTP53-infected prostate cancer cells. In our experiments, all three established prostate cancer cell lines, DU145, LNCaP, and PC3, as well as two other metastatic prostate cancer cells, ILN and DUPro-1, were similarly growth inhibited by AdWTP53 vector. Time course study of the infection of prostate cancer cells, presence of nonviable floating cells, and DNA fragmentation in AdWTP53-infected LNCaP and DU145 cells suggested a combination of cell growth arrest and cell death effects, both of which are known to be associated with wt p53 cellular function.<sup>1</sup>

The inhibitory effects of AdWTP53 on LNCaP cells containing wt p53 were unexpected. We,

therefore, have further assessed the status of p53 in LNCaP cells by utilizing an assay in which induction of the WAF1/CIP1 gene in response to DNA damaging agents is tightly correlated with the presence of functional wt p53 in cells.<sup>27</sup> The induction of WAF1 in response to doxorubicin in LNCaP cells but not in PC3 or DU145 strongly suggests the presence of wt p53 in LNCaP cells used in this study. Although a majority of reports have noticed growth inhibitory effects of wt p53 on tumor cells with mutant or no p53,<sup>1,24,25</sup> there are some studies that have also described growth inhibitory effects of wt p53 on tumor cells with endogenous wt p53.<sup>17,18,30</sup> However, it is possible that some as yet unknown function of p53 is defective in LNCaP cells studied here and further study is warranted to understand the mechanisms of inhibitory effects of AdWTP53 on LNCaP cells.

Our studies along with the earlier report describing p53 tumor suppressor effects on two prostate cancer cell lines, PC3 and TSU-PR1,<sup>10</sup> demonstrate that all six metastatic prostate cancer cell lines tested so far are growth inhibited in response to the overexpression of exogenous wt p53. Our studies with AdWTP53 vector provide support for earlier observations<sup>10</sup> and, more importantly, provide a wt p53 expression vector with a potential for future applications in gene therapy-related experiments. Recent reports have also described the gene therapy potential of recombinant adenovirus vectors expressing wt p53 in animal models of lung<sup>16</sup> and head and neck<sup>17</sup> cancers. A recent study has described an intriguing result in which an adenovirus-p53 expression vector did not inhibit the *in vitro* growth of a metastatic variant of LNCaP cells; however, the growth of these cells was inhibited *in vivo*.<sup>31</sup> In contrast, adenovirus-p53 expression vector utilized in our study exhibited a potent inhibitory effect on all the metastatic human prostate cancer cell lines tested. Another report has recently evaluated the therapeutic efficacy of a recombinant adenovirus vector expressing wt p53 in a mouse prostate reconstitution model.<sup>32</sup> Although the primary tumors derived from mouse prostate cancer cells harboring homozygous p53 mutation and exogenous *ras* and *myc* oncogenes did not show reduction in size following the injection of adenovirus vector expressing wt p53, there was a marked suppression of metastatic lesions.<sup>32</sup> The mechanism of inhibition of prostate cancer cell growth in response to AdWTP53 vector appears to involve cellular p53 pathway as evident from the up regulation of WAF1/CIP1 protein and DNA fragmentation in AdWTP53-infected prostate cancer cells noted in our study. Our preliminary results from intratumoral injections of the AdWTP53 have also shown inhibition of tumor progression



of DU145- and PC3-derived tumors in nude mice (data not shown). Additional studies utilizing animal models of prostate cancer will further characterize the in vivo antitumorigenic effects of the AdWTP53 vector.

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#### REFERENCES

- Greenblatt MS, Bennett WP, Hollstein M, and Harris CC: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 54: 4855-4878, 1994.
- Malkin D: p53 and the Li-Fraumeni syndrome. *Biochim Biophys Acta* 1198: 197-213, 1994.
- Isaacs WB, Bova GS, Morton RA, Bussemakers MJ, Brooks JD, and Ewing CM: Molecular biology of prostate cancer. *Semin Oncol* 21: 514-521, 1994.
- Moul JW, Gaddipati J, and Srivastava S: Molecular biology of prostate cancer: oncogenes and tumor suppressor genes, in Dawson NA and Vogelzang N (Eds.): *Current Clinical Oncology: Prostate Cancer*. New York, Wiley-Liss Publications, 1994, pp 19-46.
- Bookstein R, MacGrogan D, Hilsenbeck SG, Sharkey F, and Allred DC: p53 is mutated in a subset of advanced-stage human prostate cancers. *Cancer Res* 53: 3369-3373, 1993.
- Navone NM, Troncoso P, Pisters LL, Goodrow TL, Palmer JL, Nichols WW, von Eschenbach AC, and Conti CJ: p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. *J Natl Cancer Inst* 85: 1657-1669, 1993.
- Aprikian AG, Sarkis AS, Fair WR, Zhang ZF, Fuks Z, and Cordon-Cardo C: Immunohistochemical determination of p53 protein nuclear accumulation in prostatic adenocarcinoma. *J Urol* 151: 1276-1280, 1994.
- Berner A, Nesland JM, Wachre H, Silde J, and Fossa SD: Hormone resistant prostatic adenocarcinoma. An evaluation of prognostic factors in pre- and post-treatment specimens. *Br J Cancer* 68: 380-384, 1993.
- Heidenberg H, Sesterhenn IA, Gaddipati J, Weghorst M, Buzard GS, Moul JW, and Srivastava S: Alteration of the tumor suppressor gene p53 in a high fraction of hormone refractory prostate cancer. *J Urol* 154: 414-421, 1995.
- Isaacs WB, Carter BS, and Ewing CM: Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. *Cancer Res* 51: 4716-4720, 1991.
- Vogelstein B, and Kinzler KW: p53 function and dysfunction. *Cell* 70: 523-526, 1992.
- Hartwell LH, and Kastan MB: Cell cycle control and cancer. *Science* 266: 1821-1828, 1994.
- Kozarsky KF, and Wilson JM: Gene therapy: adenovirus vectors. *Curr Opin Genet Dev* 3: 499-503, 1993.
- Seth P, Fitzgerald D, Ginsberg H, Willingham M, and Pastan I: Pathway of adenovirus entry into cells, in Crowell R, and Lonberg-Holm K (Eds): *Virus Attachment and Entry into Cells*. American Society for Microbiology, Washington, DC, 1986, pp 191-195.
- Rosenfeld MA, Chu CS, Seth P, Danel C, Banks T, Yoneyama K, Yoshimura K, and Crystal RG: Gene transfer to freshly isolated human respiratory epithelial cells in vitro using a replication deficient adenovirus containing the human cystic fibrosis transmembrane conductance regulator cDNA. *Hum Gene Ther* 5: 331-342, 1994.
- Fujiwara T, Grimm EA, Mukhopadhyay T, Zhang WW, Owen-Schaub LB, and Roth JA: Induction of chemosensitivity in human lung cancer cells in vivo by adenovirus-mediated transfer of wild-type p53 gene. *Cancer Res* 54: 2287-2291, 1994.
- Clayman GL, el-Naggar AK, Roth JA, Zhang WW, Goepfert H, Taylor DL, and Liu TJ: In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. *Cancer Res* 55: 1-6, 1995.
- Katayose D, Gudas J, Nguyen H, Srivastava S, Cowan KH, and Seth P: Cytotoxic effects of adenovirus mediated wild type p53 protein expression in normal and tumor mammary epithelial cells. *Clin Cancer Res* 1: 889-897, 1995.
- Ware JL, Paulson DF, Mickey GH, and Webb KS: Spontaneous metastasis of cells of the human prostate carcinoma cell line PC-3 in athymic nude mice. *J Urol* 128: 1064-1067, 1982.
- Gingrich JR, Tucker JA, Walther PJ, Day JW, Poulton SH, and Webb KS: Establishment and characterization of a new human prostatic carcinoma cell line (DuPro-1). *J Urol* 146: 915-919, 1991.
- Janat MF, Srivastava S, Devadas K, Chin GA, Pirollo KF, Chang EH: Inhibition of retinoblastoma protein phosphorylation by the combined effect of interferon- $\gamma$  and tumor necrosis factor- $\alpha$ . *Mol Cell Diff* 2: 241-253, 1994.
- Srivastava S, Tong YA, Devadas K, Zou ZQ, Sykes VW, Chen Y, Blattner WA, Pirollo K, and Chang EH: Detection of both mutant and wild-type p53 protein in normal skin fibroblasts and demonstration of a shared "second-hit" on p53 in diverse tumors from a cancer-prone family with Li-Fraumeni syndrome. *Oncogene* 7: 987-991, 1992.
- Towbin H, Staehelin T, and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels into nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76: 4350-4354, 1979.
- Baker SJ, Markowitz S, Fearon ER, Willson JK, and Vogelstein B: Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 249: 912-915, 1990.
- Oren M: p53: the ultimate tumor suppressor gene? *FASEB J* 6: 3169-3176, 1992.
- el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, and Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817-825, 1993.
- el-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, et al: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 54: 1169-1174, 1994.
- Sanda MG, Ayyagari SR, Jaffee EM, Epstein JI, Clift SL, Cohen LK, Dranoff G, Pardoll DM, Mulligan RC, and Simmons JW: Demonstration of a rational strategy for human prostate cancer gene therapy. *J Urol* 151: 622-628, 1994.
- Vieweg J, Rosenthal FM, Bannerji R, Heston WD, Fair WR, Gransbacher B, and Gilboa E: Immunotherapy of prostate cancer in Dunning rat model: use of cytokine gene modified tumor vaccine. *Cancer Res* 52: 1760-1765, 1994.
- Cajot JF, Anderson MJ, Lehman TA, Shapiro H, Briggs AA, and Stanbridge EJ: Growth suppression mediated by transfection of p53 in Hut292DM human lung cancer cells expressing endogenous wild type p53 protein. *Cancer Res* 52: 6956-6960, 1992.
- Ko AG, Gotoh A, Thalman GN, Cleutjens C, Trapman J, Ling-Jun H, Zhang WW, Chung LK, and Kao C: Gene therapy for treatment of androgen independent and PSA producing human prostate cancer. *Basic and Clinical Aspects of Prostate Cancer Meeting*, Palm Springs, Abstract C-21, Dec 1994.
- Sehgal I, Eastham JA, Zhang WW, Hall S, Park SH, Kadmon D, Scardino PT, and Thompson TC: Adenovirus-mediated p53 gene transfer in experimental prostate cancer. Abstract 321, AUA Ninetieth Annual Meeting April 23-28, 1995. *J Urol* 153: 309A, 1995.



## Growth Suppression Mediated by Transfection of p53 in Hut292DM Human Lung Cancer Cells Expressing Endogenous Wild-Type p53 Protein C57

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### Abstract

This study was undertaken to analyze the effect of wild-type p53 transfection on the growth potential of a human lung cancer cell line Hut292DM expressing endogenous wild-type p53. Transfection efficiencies obtained with either the wild-type or a mutant p53 complementary DNA revealed a significant decrease in the number of colonies obtained with the wild-type p53 as compared to the mutant p53 complementary DNA (27%) or control vector DNA only (20%), suggesting that wild-type p53 inhibited the growth of Hut292DM cells. A series of wild-type and mutant p53 transfection clones were then analyzed for the presence and expression of the exogenous p53 gene. Polymerase chain reaction amplification revealed that 98% of mutant p53 transfection clones analyzed contained the exogenous p53 gene as opposed to 47% for wild-type p53 clones. The majority of mutant p53 clones expressed high levels of exogenous p53 mRNA and protein as analyzed by Northern and Western blots, respectively. In contrast, all wild-type p53 clones analyzed failed to express exogenous p53 mRNA transcript or protein of a normal size. Aberrant-size p53 mRNA was detected in two wild-type p53 clones (X833.W2 and W18), and Western blot analysis revealed that these clones expressed truncated p53 proteins (M<sub>r</sub> 45,000 and 33,000 respectively). No difference in proliferation rates *in vitro* or in tumorigenic potential in nude mice were observed between mutant p53 clones or control cell lines. In contrast, a wild-type p53 clone (X833.W2) exhibited a significantly reduced tumorigenic potential in nude mice, whereas its *in vitro* proliferation rate was comparable to parental Hut292DM cells. The data indicate that exogenous expression of wild-type p53 is incompatible with Hut292DM lung cancer cell proliferation *in vitro* and suggest that p53-mediated growth control *in vitro* and *in vivo* may be dissociated and exerted by separate domains of the p53 protein.

### Introduction

Since its discovery approximately a decade ago, the p53 tumor suppressor gene has attracted a great deal of attention in cancer research, partly due to its capacity to prevent uncontrolled growth of cancer cells and due to the realization that most types of human cancers studied, including lung, display some form of alteration of the p53 gene (1). The molecular mechanisms by which p53 may regulate cell cycle progression and therefore prevent tumor cell growth are poorly resolved at the present time. A variety of human tumor cells have now been shown to be growth arrested when transfected with the p53 tumor suppressor gene (2-5), and the cell cycle progression of these recipient cells is blocked in the G<sub>1</sub>-S transition phase (3, 6). However, this effect is seen only when the cancer cells contain mutated p53 or are null for expression. To date, reports

of the introduction of wild-type p53 cDNA<sup>2</sup> into tumor cells containing endogenous wild-type p53 have shown that there is no effect on *in vitro* growth (2, 4).

The product of the p53 gene is a 393-amino-acid-long protein, primarily localized in the cell nucleus, where it may interact with genomic DNA sequences to regulate the cell cycle in two ways. The carboxy-terminal portion of the protein is associated with DNA binding, and a consensus DNA sequence which binds to p53 has now been defined that shares homology with DNA origins of replication (7, 8). The amino-terminal domain functions as a transcriptional modulator, possibly involved in the repression of nuclear oncogene transcription (9). In addition, p53 may control cell growth by binding to, and interacting with, nuclear proteins critically involved in cell cycle regulation such as the cyclin-dependent cdc2 kinase (10) or the murine double minute 2 oncogene product (11). Recent studies have shown that many cancer cells no longer express a normal p53 protein, the result of both alleles being inactivated by a combination of single allele deletion and point mutation of the remaining allele (12, 13). However, a significant proportion of human cancers arise in which no detectable alteration in p53 expression is observed, seemingly precluding any role of the p53 tumor suppressor gene in the natural development of these tumors. The present study was undertaken to determine whether human lung cancer cell growth could be influenced by transfection of the p53 gene. To our knowledge, we are the first to report growth suppression induced by high level expression of exogenous wild-type p53 in lung cancer cells expressing normal endogenous p53 protein.

### Materials and Methods

**Transfection.** Human lung cancer Hut292DM cells were kindly provided by C. Harris (14) and were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Transfections were performed by the polybrene-dimethyl sulfoxide technique as described (15) using plasmid constructs obtained from B. Vogelstein: pCMVneoBam, a eukaryotic expression vector containing the cytomegalovirus constitutive promoter and the neomycin resistance gene under control of the simian virus 40 promoter; pC53SN3, derived from pCMVneo by insertion of a full-length human wild-type p53 cDNA under control of the cytomegalovirus promoter; and pC53SCX3, which contains a single point mutation in the p53 cDNA at codon 143 (substitution of alanine to valine). Hut292DM cells (2 × 10<sup>6</sup>) were transfected with 20 µg of plasmid DNA, and following 3-4 weeks of selection in medium containing 1100 µg/ml geneticin, colonies were either stained with 5% Giemsa to assess transfection efficiency or cloned and expanded for further analysis.

**Molecular Analysis of p53 Expression.** Standard nucleic acid procedures were performed as described (15). PCR analysis was performed

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<sup>2</sup> The abbreviations used are: cDNA, complementary DNA; PCR, polymerase chain reaction.

using DNA extracts obtained by the rapid detergent method described (4). The two primers used for p53 cDNA amplification (p53.1: 5'-CAC GAC GGT GAC ACG CTT CCC TG-3'; p53.2: 5'-GTC CTG GGT GCT TCT GAC GCA CAC-3') are complementary to 5' and 3' flanking sequences of the p53 coding region, respectively, and yield a 1.2-kilobase PCR product. Southern and Northern blot analyses were performed using genomic DNA extracts digested with *Bam*HI or total cytoplasmic RNA extracted as described (16). Nucleic acids were separated on 1% agarose gels (DNA) or 1.2% agarose-formaldehyde denaturing gels (RNA) and transferred to nitrocellulose membranes. All hybridizations were performed using a 1.8-kilobase full-length p53 cDNA probe derived from pC53SN (following *Bam*HI digestion) and radiolabeled by the random primer method. Protein extraction and Western blot analysis were performed as described (17). Protein extracts were isolated from 50% confluent cells using RIPA buffer for cell lysis, and the protein concentration was determined by a modified Lowry colorimetric assay (DC protein assay kit; BioRad, Richmond, CA). Equal amounts of protein extracts (100 µg) were denatured (5 min at 100°C in presence of 0.1 M dithiothreitol), separated through 10% polyacrylamide-sodium dodecyl sulfate gels, and transferred to nitrocellulose membranes. Immunoblotting was performed using the monoclonal antibody PAb 1801 (Oncogene Science, Manhasset, NY) diluted 1:500 as the primary antibody and a horseradish peroxidase-conjugated goat anti-mouse IgG (BioRad) diluted 1:2000 as the secondary antibody. Detection of p53 antigen was performed using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and following the manufacturer's recommendations.

**Cell Growth Analysis and Tumorigenicity Assay.** Cells ( $1.5 \times 10^5$ ) were plated in 35-mm tissue culture wells, and at various time points cells from duplicate wells were counted. Culture medium was changed 3 times each week. Tumorigenicity was assessed by inoculating  $5 \times 10^4$  cells at 6 sites into 4–6-week-old athymic nude mice (one inoculation per animal). Tumors were measured with linear calipers at regular intervals to compare growth properties between the different cell lines.

## Results and Discussion

Three separate transfection experiments were performed in an attempt to express exogenous p53 cDNA (wild-type and mutant versions) in Hut292DM human lung cancer cells. Transfection efficiencies (see Table 1) from all three experiments indicate a significant decrease in the number of geneticin-resistant colonies obtained with the wild-type p53 cDNA as compared to the vector construct alone. In contrast, the mutant p53 cDNA did not appear to affect the transfection efficiency. These results suggested that expression of exogenous wild-type p53 inhibits Hut292DM cell growth or that high levels of expression are incompatible with sustained proliferation of these cells. A detailed molecular analysis of p53 expression in a series of transfection clones was then undertaken to gain more insight into the possible role of p53 in the control of Hut292DM tumor cell growth. PCR analysis of p53 cDNA was performed on a series of mutant and wild-type p53 transfection clones derived from two separate experiments (X813 and X833). The majority

of X833 mutant p53 clones analyzed displayed a 1.2-kilobase PCR amplification product which contains the entire p53 coding region (16 of 18, or 89%) whereas only 47% (8 of 17) of the wild-type p53 transfection clones displayed a p53 cDNA-related PCR product (Fig. 1A). Interestingly, one of these clones (X833.W2) yielded an unusually large PCR product of 1.8 kilobases which indicates a probable rearrangement of the p53 coding region either by insertion of foreign sequences or by partial duplication of the introduced p53 cDNA. Southern blot analysis of PCR-positive clones confirmed the presence of exogenous p53 cDNA sequences in all X833 mutant p53 clones analyzed (8 of 8) and in most (7 of 9) wild-type p53 clones (data not shown). However, only a few of the wild-type clones (3 of 9, or 33%) displayed the expected 1.8-kilobase hybridization signal, the other clones being associated with larger-size hybridization signals corresponding to plasmid DNA rearrangement. In contrast, nearly all mutant clones (6 of 7, or 86%) displayed the expected 1.8-kilobase band, which suggests that the cDNA has not been rearranged upon integration. These results not only confirm the PCR analysis carried out on the mutant p53 transfectants but also show a further decrease in the number of clones harboring a normal copy of wild-type p53 cDNA.

Northern blot analysis of these transfection clones was then performed to examine p53 mRNA expression (Fig. 1B). All transfection clones analyzed, as well as parental Hut292DM cells, express a 2.8-kilobase band which corresponds to endogenous p53 mRNA. This band appears to be of comparable intensity for all clones analyzed and therefore indicates equal RNA loading of all samples. The exogenous p53 mRNA can be differentiated in this analysis from endogenous mRNA due to its smaller size of 2.65 kilobases. The majority (5 of 8, or 63%) of the mutant p53 clones analyzed expressed very high levels of the 2.65-kilobase exogenous mRNA species. In contrast, no normal-size transcript characteristic of exogenous p53 was detected in any of the wild-type p53 clones analyzed. The same analysis performed on a series of separate transfection clones (experiment X813) was also performed and resulted in similar findings. Briefly, PCR analysis for the presence of p53 cDNA was positive for the majority of mutant X813 transfection clones analyzed (7 of 12, or 58%) whereas only two wild-type X813 clones were positive in this assay (2 of 12, or 17%). Northern blot analysis showed high levels of expression of the exogenous p53 transcript in most of the X813 mutant p53 transfection clones analyzed, whereas the X813 wild-type p53 clones positive by PCR analysis failed to express the exogenous transcript.

Two of the wild-type p53 transfection clones (X833.W2 and W18), however, did express high levels of aberrant p53 mRNA transcripts, a finding which prompted us to analyze whether these transcripts might be translated. Fig. 1C shows the expression of p53 protein by clones previously analyzed for p53 mRNA expression. As with the Northern blot analysis, p53 antigen was easily detected in most mutant p53 clones analyzed (7 of 8, or 88%), which is in agreement with the prolonged half-life of mutant forms of p53 compared to that of wild-type. In contrast, a very faint band corresponding to endogenous p53 protein was detected in parental Hut292DM cells as well as in all of the wild-type clones. No increase in the expression level of this *M<sub>r</sub>* 53,000 band was observed in the wild-type p53 clones, which correlates with the absence of normal-size exogenous transcript detected by Northern analysis. Several wild-type clones did, however, express high levels of truncated forms of

Table 1 Transfection efficiencies of wild-type and mutant p53 expression vectors in Hut292DM cells

Three separate transfection experiments (X813, X832, X833) were performed using Hut292DM cells and the plasmid DNA constructs pC53SN and pC53SCX3, which contain a human p53 cDNA (wild-type or mutated at codon 143, respectively) under transcriptional control of the human cytomegalovirus promoter, as well as pCMVneoBam (vector DNA only). Transfection efficiencies are expressed as the total number of geneticin-resistant colonies obtained using 20 µg plasmid DNA and  $2 \times 10^6$  cells and after 4 weeks of selection.

Experiment	pC53SN3 (wild-type)	pC53SCX3 (mutant)	pCMVneoBam (vector)
X813	115	600	720
X832	55	240	300
X833	780	1900	2520

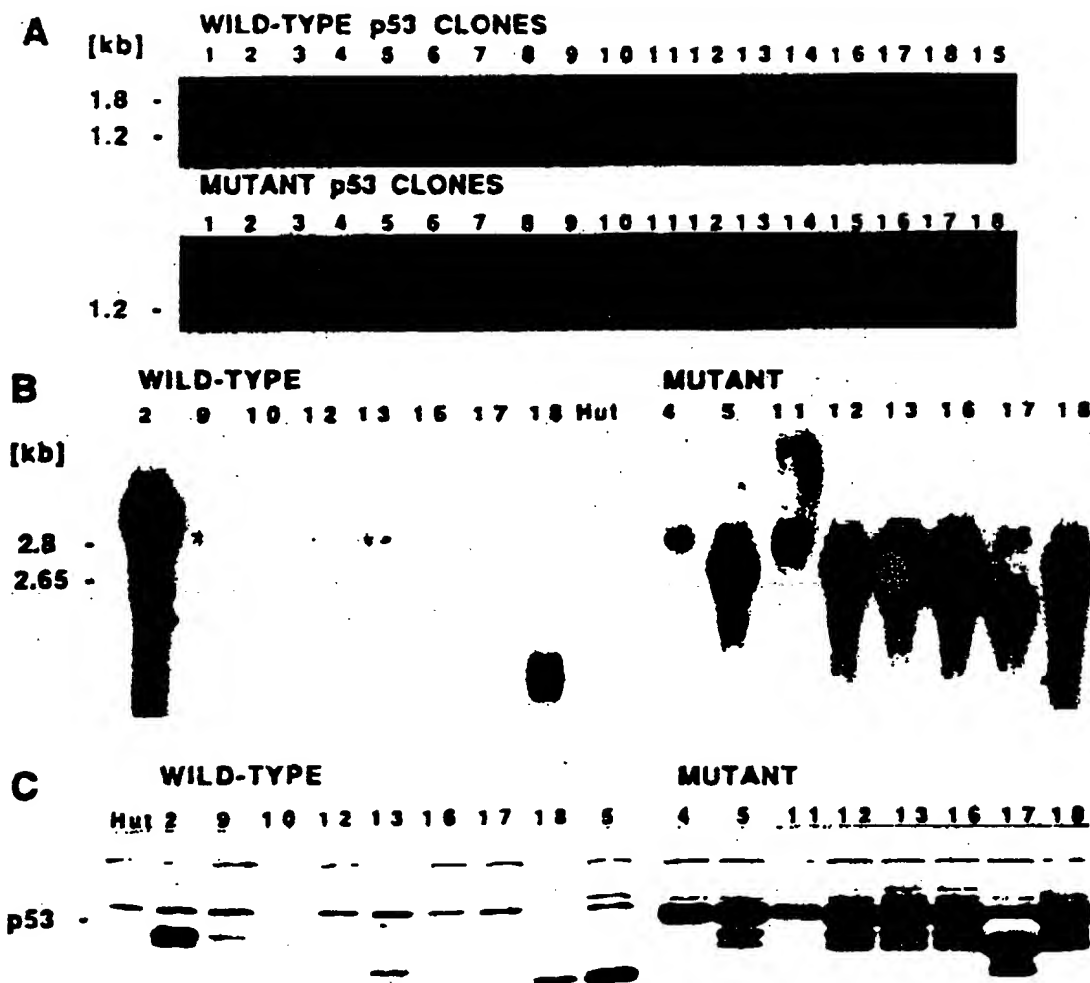


Fig. 1. Analysis of exogenous p53 expression in a series of wild-type and mutant p53 transfection clones derived from Hut292DM cells (transfection experiment: X833). A, PCR amplification of a 1.2-kilobase fragment corresponding to the full-length coding region of p53 cDNA. Top, wild-type p53 transfection clones; bottom, mutant p53 clones. B, Northern blot analysis of wild-type (left) and mutant p53 (right) transfection clones as well as control parental Hut292DM cells (Lane Hut). Endogenous p53 mRNA migrates at a position corresponding to 2.8 kilobases and may be differentiated from exogenous p53 mRNA (2.65 kilobases). C, Western blot analysis of p53 antigen using the same cells as in B. The position of migration of the normal p53 is indicated.

p53 protein. These truncated p53 variants appear to be related to the aberrant mRNA transcripts expressed by these clones. In particular, clones X833.W2 and W18, which were associated with a larger and smaller size mRNA transcript, respectively, both appear to synthesize, respectively, truncated  $M_r$  ~45,000 and ~33,000 p53 proteins. Two additional bands of higher molecular weight (approximately 55,000 and 75,000) were detected in all samples analyzed; these probably represent common epitopes shared by proteins unrelated to p53 but recognized by the monoclonal antibody used in this assay.

Selected clones were further analyzed for their growth properties *in vitro* (Fig. 2). The growth rates (e.g., doubling times) and saturation densities of various mutant p53 clones did not vary significantly from that of parental Hut292DM cells. Furthermore, wild-type p53-transfected cells shown to express high levels of truncated p53 protein exhibited similar *in vitro* growth properties as parental Hut292DM cells or cells transfected with the control vector only (data not shown). The results, however,

were different when these transfected clones were assayed *in vivo* for their tumorigenic potential (Fig. 3). A particular wild-type p53 transfection clone (X833.W2) consistently exhibited a reduced tumorigenic potential compared to parental or vector transfected cells. Interestingly, X833.W2 cells were shown by Western blot analysis to express what appears to be a mildly truncated form of the p53 protein with a molecular weight of approximately 45,000. Moreover, all other forms of truncated p53 protein of smaller size did not appear to affect the tumorigenic potential of Hut292DM transfection clones. Various mutant p53 transfection clones, tested under identical experimental conditions, displayed a tumorigenic potential similar to that of parental or vector transfected Hut292DM cells (data not shown).

The results of the molecular analysis of p53 expression in wild-type and mutant p53 transfection clones suggest that growth suppression is mediated by high expression levels of wild-type p53 in this human lung cancer cell line. Indeed, the

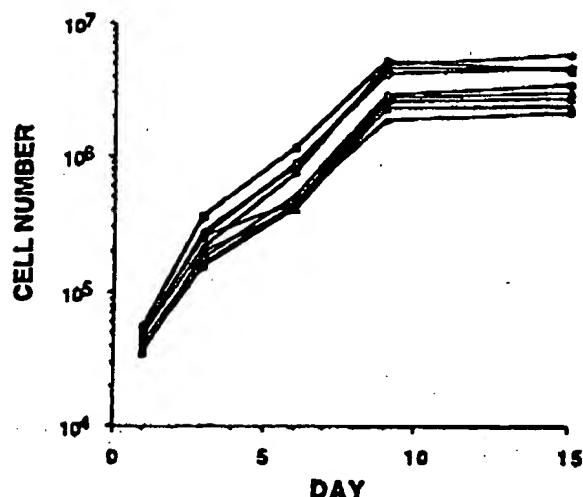


Fig. 2. *In vitro* growth rate analysis of Hut292DM parental cells as well as four mutant p53 and four control-vector-only transfection clones. Cell lines analyzed are: Hut292DM (X); mutant p53 transfection clones, X833.M12 (■), M13 (●), M16 (Δ), M18 (○); vector-only clones, X833.N1 (□), N2 (○), N4 (△), N6 (·). For details on the experimental procedure followed, see "Materials and Methods."

clonal analysis, repeated twice on two separate transfection experiments, consistently failed to identify any wild-type transfection clones expressing a normal exogenous p53 protein, whereas exogenous mutant p53 appeared to be stably expressed at high levels in nearly all clones analyzed. We conclude from these results that expression of exogenous wild-type p53 is incompatible with the sustained proliferation of Hut292DM cells, a finding consistent with the transfection efficiencies obtained with the wild-type and mutant cDNA constructs (see Table 1). These results also demonstrate that a single point mutation at codon 143 (a transition from an alanine to a valine residue) results in the apparent loss of function of p53 with respect to its growth-inhibitory properties for Hut292DM cells, since no difference in transfection efficiency was noted using either the mutant p53 cDNA or the control vector. Several studies involving human colon, breast, and lung cancer cells (2, 4, 5) have also shown that restoration of normal p53 protein expression in otherwise defective cell lines (either null for p53 expression or carrying a p53 mutation) severely affected the growth capacity of these model cell lines. This indicates that the loss of function resulting from p53 alterations may represent a critical step in human carcinogenesis and could lead to some form of gene therapy based on the restoration of expression of p53 in defective cell lines. However, previous efforts to influence the growth potential of tumor cells expressing endogenous wild-type p53 by transfection of the p53 gene were not successful (2, 4), although the reason for this failure is poorly understood. We now demonstrate that expression of exogenous wild-type p53 may lead to the growth arrest of highly proliferative tumor cells constitutively expressing endogenous wild-type p53. The status of the p53 gene in this cell line was analyzed previously by full-length sequencing of the entire coding region (14). Hut292DM cells do not contain a p53 mutation, consistent with our present Western blot analysis showing the expression of trace amounts of p53 protein in these cells. At present, we may only speculate as to how exogenous expression of p53 results in growth suppression of Hut292DM cells, perhaps by overwhelming the normal regulatory mechanism of p53 function. To date, possible mechanisms include p53 phosphoryla-

tion or interaction with another protein (18). An alternative explanation for the capability of additional exogenous wild-type p53 to inhibit cell growth may reside in the very short half-life associated with wild-type p53 protein as compared with the prolonged half-life of mutant forms of the protein. It may be argued that specific proteases are involved in the degradation and elimination of p53 function throughout the cell cycle and that when a critical level of p53 protein synthesis is reached these proteolytic events are no longer able to control overexpression of wild-type p53. Furthermore, a negative feedback mechanism for p53 expression has recently been described whereby p53 represses the transcriptional activity of the p53 natural promoter (19). Evidently, this negative regulatory mechanism was not effective in our transfection studies, which used a cytomegalovirus heterologous promoter for exogenous p53 expression. We do not necessarily favor a mere toxic effect elicited by high levels of wild-type p53 expression, as previous studies using identical expression vectors have shown that certain tumor cells tolerate such levels of expression without any observed effect (2, 4). Therefore, our present work extends the scope of the potential effectiveness of wild-type p53 to control tumor growth to recipient cells that contain no apparent defect in endogenous wild-type p53 expression. As our knowledge of p53 regulatory mechanisms increases it will be of interest to compare our results with those of other model systems, which may help understand why Hut292DM cells are sensitive to exogenous wild-type p53 expression.

Our data also indicate that although expression of exogenous p53 is incompatible with Hut292DM cell growth *in vitro*, we were able to isolate stable transfection clones expressing various truncated forms of p53 protein. Cells expressing high levels of truncated p53 protein were not, however, affected in their *in vitro* growth properties. This may indicate that a critical domain of the molecule which mediates tumor cell growth control *in vitro* has been deleted from the final translation product, possibly as a result of gene rearrangement or point mutation leading to a stop codon. The lack of any apparent inhibition of

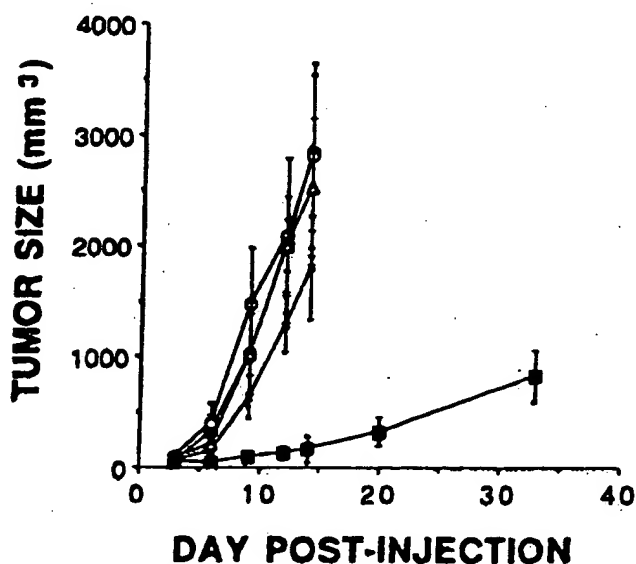


Fig. 3. Tumorigenicity assay in nude mice of Hut292DM parental cells as well as wild-type and mutant p53 transfection clones. Cell lines analyzed are: Hut292DM (X); wild-type p53 transfection clone X833.W2 (■); mutant p53 clones X833.M13 (●), and M18 (○); and vector-only transfection clone X833.N2 (Δ). For details on the experimental protocol, see "Materials and Methods."

growth *in vitro* displayed by cells expressing various truncated p53 protein was in sharp contrast to the tumorigenic potential of X833.W2 cells, which synthesize a high level of a M, 45,000 p53 truncated protein and consistently displayed growth inhibition *in vivo*. Although our observations to date are based on a single clonal event, the results suggest that growth control mechanisms mediated by p53 differ *in vitro* from *in vivo* situations and that separate domains of the p53 molecule may be responsible for these effects. We are now in the process of sequencing these altered forms of p53 in order to obtain a better definition of the domains possibly involved in growth regulation observed *in vitro* and *in vivo*. Although we are only beginning to understand the mechanisms by which the p53 tumor suppressor gene may control abnormal proliferation of cancer cells, this study provides original evidence that a human lung cancer cell line expressing endogenous wild-type p53 can be growth inhibited by high levels of p53 expression. These results should also provide useful information on the domain-function organization of the p53 protein.

## References

- Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. *Science* (Washington, DC), 253: 49-53, 1991.
- Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. V., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* (Washington DC), 249: 912-915, 1990.
- Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. R., and Ulrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA*, 87: 6166-6170, 1990.
- Casey, G., Lu-Hyueh, M., Lopez, M. E., Vogelstein, B., and Stanbridge, E. J. Growth suppression of human breast cancer cells by introduction of a wild-type p53 gene. *Oncogene*, 6: 1791-1797, 1991.
- Takahashi, T., Carbone, D., Takahashi, T., Nau, M. N., Hida, T., Linnoila, I., Ueda, R., and Minna, J. D. Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. *Cancer Res.* 52: 2340-2343, 1992.
- Mercer, W. E., Nelson, D., DeLeo, A. B., Old, L. J., and Baserga, R. Microinjection of monoclonal antibody to protein p53 inhibits serum-induced DNA synthesis in 3T3 cells. *Proc. Natl. Acad. Sci. USA*, 79: 6309-6312, 1982.
- El-Deiry, W., Kern, S. E., Pietenpol, J. A., Kluzier, K. W., and Vogelstein, B. Definition of a consensus binding site for p53. *Genetics*, 1: 45-49, 1992.
- Funk, W. D., Pak, D. T., Karns, R. H., Wright, W. E., and Shay, J. W. A transcriptionally active DNA-binding site for human p53 protein complex. *Mol. Cell. Biol.*, 12: 2866-2871, 1992.
- Pickles, S., and Jang, S. K. Presence of a potent transcription activating sequence in the p53 protein. *Science* (Washington DC), 249: 1046-1049, 1990.
- Blachuff, J. R., Friedman, P. N., Marshak, D. R., Prives, C., and Beach, D. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. *Proc. Natl. Acad. Sci. USA*, 87: 4766-4770, 1990.
- Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, 69: 1237-1245, 1992.
- Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Milburn Jessup, J., vanTuinen, P., Ledbetter, D. H., Barber, D. F., Nakamura, Y., White, R., and Vogelstein, B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* (Washington DC), 244: 217-221, 1989.
- Nigro, J. M., Baker, S. J., Preisinger, A. C., Milburn Jessup, J., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Bayle, S., DeVilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. Mutations in the p53 gene occur in diverse human tumour types. *Nature* (Lond.), 342: 705-708, 1989.
- Lehman, T. E., Bennett, W. P., Metcalf, R. A., Welsh, J. A., Ecker, J., Modali, R. V., Ulrich, S., Romano, J. W., Appella, E., Testa, J. R., Gerwin, B. I., and Harris, C. C. p53 mutations, ras mutations, and p53-hes shock 70 protein complexes in human lung carcinoma cell lines. *Cancer Res.*, 51: 4090-4096, 1991.
- Sambrook, J. E., Fritsch, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
- Gough, N. M. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Anal. Biochem.*, 173: 93-95, 1988.
- Harlow, E., and Lane, D. *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.
- Montenarh, M. Functional implications of the growth-suppressor/oncoprotein p53 (review). *Int. J. Oncol.*, 1: 37-45, 1992.
- Ginsberg, D., Mechta, F., Yaniv, M., and Oren, M. Wild-type p53 can down-modulate the activity of various promoters. *Proc. Natl. Acad. Sci. USA*, 88: 9979-9983, 1991.

## Cytotoxic Effects of Adenovirus-mediated Wild-Type p53 Protein Expression in Normal and Tumor Mammary Epithelial Cells

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### ABSTRACT

To evaluate the effects of the wild-type p53 expression in normal and tumor cells, we have constructed a recombinant adenovirus vector (E1 minus) expressing human wild-type p53 cDNA (AdWtp53). Infection of normal and tumor cells of lung and mammary epithelial origin with AdWtp53 resulted in high levels of wild-type p53 expression. Production of p53 protein following infection was dependent on the dose of AdWtp53 with maximum amounts of p53 produced following infection with 50 plaque-forming units/cell. AdWtp53 infection inhibited the growth of all human cell lines studied. However, tumor cells that were null for p53 prior to infection (H-358 and MDA-MB-157) and tumor cells that expressed mutant endogenous p53 protein (MDA-MB-231 and MDA-MB-453) were more sensitive to AdWtp53 cytotoxicity than cells that contained the wild-type p53 (MCF-7, MCF-10, 184B5, and normal mammary epithelial cells). All cells exhibited WAF1/Cip1 mRNA and protein induction following AdWtp53 infection. AdWtp53-induced cytotoxicity of human tumor cell lines expressing mutant p53 was mediated by apoptosis as revealed by nucleosomal DNA fragmentation analysis. No detectable nucleosomal DNA fragmentation was observed following AdWtp53 infection of human cells expressing wild-type p53. These data suggest that endogenous p53 status is a determinant of AdWtp53-mediated cell killing of human tumor cells.

### INTRODUCTION

The tumor suppressor gene p53 is apparently the most frequently altered gene analyzed in human tumors, including those from breast and lung (1-3). Recently, there has been an increasing interest in elucidating the mechanisms by which p53 mediates its functions in normal cells, how various mutations in p53 are responsible for aberrant cell growth (1, 4), and the possibility of using wild-type p53 in gene therapy (5). It is therefore important to understand the biological consequences of the wild-type p53 overexpression in both normal and tumor

cells. Different approaches have been used to study the effects of p53 expression in cells, including exposure of cells to UV radiation and DNA damaging agents (6-9), both of which have been shown to induce increased expression of cellular p53. Alternative genetic approaches have also been used, including introduction of a temperature-sensitive mutant of p53 (10-12) or gene knock-out experiments (13) to alter intracellular p53 expression and function. There is compelling evidence that wild-type p53 can negatively influence cell growth by causing G<sub>1</sub> arrest (8, 10) and/or by inducing apoptosis (6, 7, 11, 12, 14, 15). In cells expressing mutant p53, these effects of wild-type p53 are abrogated, resulting in abnormal cell growth (1, 4, 15).

One approach to understanding the role of p53 protein is to utilize p53 expression vectors capable of producing high levels of p53 protein in cells. This strategy not only allows the study of the role of p53 in the control of regulation of cell growth in both normal and malignant cells, but also has implications in gene therapy for cancers which are null for p53 or express mutated p53. Although plasmids and retroviruses have been used to express p53 protein (4, 14, 16), the efficiency of these transfection techniques is generally low. To study the effects of wild-type p53 expression in normal and transformed epithelial cells, we have generated a replication-deficient Ad<sup>5</sup> containing a human wild-type p53 cDNA (AdWtp53). The choice of adenovirus vector was made because adenovirus-based vectors can grow to high titers (17, 18), are internalized into cells with an efficient receptor-mediated endocytosis (19, 20), are replication incompetent (21, 22), and express a transgene to high levels in epithelial cells (20, 22). We have used this vector to express wild-type p53 protein in tumor and normal cells with different intrinsic p53 status (null, mutant, or wild type) to (a) examine the effects of high levels of p53 on the growth properties of normal and tumor cells, (b) examine the effects of p53 expression on the induction of WAF1/Cip1 and mdm2 gene expression, and (c) evaluate the role of apoptosis in p53-mediated cytotoxicity. These studies indicate that an adenovirus vector expressing wild-type p53 is markedly cytotoxic to tumor cells that have null or mutant p53 expression, and that this vector can provide a useful tool to study the precise molecular mechanisms by which p53 mediates its effects in normal and transformed cells.

### MATERIALS AND METHODS

**Construction of an AdWtp53.** AdWtp53 was constructed using cotransfection of shuttle vector pDK10 containing the wild-type p53 expression cassette and a plasmid pJM17 containing the adenovirus type 5 genome. The shuttle vector

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<sup>3</sup> The abbreviations used are: Ad, adenovirus; FBS, fetal bovine serum; EGF, epidermal growth factor; NMEC, normal mammary epithelial cell; pfu, plaque-forming unit; Adr<sup>r</sup>, Adriamycin resistant; IC<sub>50</sub>, 50% inhibitory concentration;  $\beta$ -gal,  $\beta$ -galactosidase.



pDK10 was constructed by inserting the human cytomegalovirus immediate early promoter and enhancer, a 1.7-kb *Xba*I fragment of human p53 cDNA (23), the SV40 small T intron, and SV40 polyadenylation signal into the *Clu*I site of plasmid pXCX2 (18). Plasmid pDK10 was cotransfected with pJM17 (Ref. 24; kindly provided by F. Graham, McMaster University, Hamilton, Ontario, Canada) into the transformed human embryonic kidney cell line 293 (ATCC CRL1573) by calcium phosphate-mediated gene transfer technique (Refs. 18 and 25; GIBCO-BRL, Gaithersburg, MD). The day following transfection, the medium was replaced with a 1X MEM (GIBCO-BRL) containing 1% sea plaque agarose gel (FMC, Rockland, ME) and 10% FBS (GIBCO-BRL), and the cells were incubated at 37°C. Every five days 2 ml MEM containing 1% sea plaque agarose gel and 10% FBS were added to the top of the cells until plaques were observed. Isolated plaques were picked and subjected to another cycle of infection in 293 cells as described previously (18, 25).

Purified recombinant AdWtp53 viruses were assayed for the absence of E1a and the presence of p53 sequences using PCRs (26). Cell lysates were prepared 24 h following infection with adenoviral vector using guanidine thiocyanate solution, and aliquots were used for PCR analysis. For E1a analysis, the primers used were 5'-TCTTGAGTGCCAGCGAGTAG-3' and 5'-CAAGGTTTGGCATAGAAACC-3'. For p53, one primer (5'-GTTGGCTCTGACTGTACC-3') was selected from exon 7, and the downstream primer 5'-GTTCCTCCAGTAGAT-TACC-3' was selected from the exon 8. This combination of primers can differentiate the PCR product of the endogenous genomic p53 gene from the viral-associated p53 gene. AdWtp53 was propagated in 293 cells grown in monolayers, purified by two cesium chloride density gradients, dialyzed against a buffer containing 10% glycerol, 1 mM MgCl<sub>2</sub> (pH 7.5), and stored at -70°C as described previously (20).

Control adenovirus vectors used in this study were: Ad.RSVβgal, an adenovirus vector containing β-galactosidase gene (27), and AdControl, an adenovirus vector dl312 containing no insert (Ref. 21; kindly provided by T. Shenk, Princeton, NJ).

**Cell Culture.** Breast cancer cell lines [MDA-MB-231 (ATCC HTB26), MCF-7 (kindly provided by R. Buick, University of Toronto, Toronto, Ontario, Canada), and Adr<sup>R</sup> MCF-7 (28)] were cultured in α-MEM (GIBCO-BRL) supplemented with 10 mM HEPES, 2 mM glutamine, 0.1 mM nonessential amino acids, 10% FBS, 1 ng/ml EGF, and 2 μg/ml insulin (29). H-358, a lung cancer cell line (kindly provided by J. Minna, University of Texas, Dallas, TX), and MDA-MB-453 cells, a breast cancer cell line (ATCC HTB131), were grown in RPMI 1640 containing 10% FBS. MDA-MB-157 (ATCC HTB 24), a breast cancer cell line, was grown in improved minimum essential medium (IMEM) (GIBCO-BRL) supplemented with 10% FBS and 0.5% Redu-Ser II (Upstate Biotechnology Inc., Lake Placid, NY). NMECs derived from reduction mammary glands (CC-2016; Clonetics Corp., San Diego, CA), and 184B5 cells, immortalized mammary epithelial cells (ATCC CRL10317), were cultured in mammary epithelial basal medium (Clonetics Corp.) supplemented with 1X vitamins, 0.5% FBS, 20 ng/ml EGF, 5 μg/ml hydrocortisone, and 52 μg/ml bovine pituitary extract (29). Immortalized MCF10 cells (kindly provided by S.

Brooks, Michigan Cancer Foundation, Detroit, MI) were cultured in DMEM/F12 (GIBCO-BRL) supplemented with 2.5% horse serum (GIBCO-BRL), 10 mM HEPES (Calbiochem, La Jolla, CA), 2 mM glutamine (Biofluids, Rockville, MD), 0.1 mM nonessential amino acids (GIBCO-BRL), 20 ng/ml EGF (Upstate Biotechnology), 10 μg/ml insulin (Boehringer Mannheim, Indianapolis, IN), and 0.5 μg/ml hydrocortisone. 293 (ATCC CRL 1573), an adenovirus-transformed human embryonic kidney cell line, was cultured in improved MEM (Biofluids) supplemented with 2 mM glutamine (Biofluids), 2.5 μg/ml Fungizone (Biofluids), 100 units/ml penicillin, 100 μg/ml streptomycin (100xPen-Strep; Biofluids), and 10% FBS.

**Effect of AdWtp53 on Cell Growth.** To study the effect of adenovirus vectors on cell growth,  $5 \times 10^4$  cells were plated in each well of 6-well dishes. After 24 h, cells were exposed to AdWtp53 or AdControl (10 pfu/cell) in medium containing 2% FBS. After an incubation of 2 h at 37°C, serum concentration in the medium was raised to 10% and incubations continued at 37°C. Cells were trypsinized on each day and counted using a hemacytometer. Cytotoxicity of adenovirus vectors was assessed using a colorimetric assay as described previously (30). Briefly, 500 cells were plated in each well of 96-well plates and incubated for 24 h. Cells were then exposed to the appropriate cell growth medium except that the concentration of the serum (if it was a component of the growth medium) was reduced to 2%. Different doses of adenovirus vectors were included in the incubation medium (several 5-fold dilutions). After 2-h incubation at 37°C, the serum concentration was increased to 10%, and the cells were incubated for 7 days at 37°C. Cells were fixed by the addition of ice-cold 50% trichloroacetic acid (added onto the top of the medium in each well to a final concentration of 10%), incubated at 4°C for 1 h, washed five times with water, and then air dried. Trichloroacetic acid-fixed cells were stained for 20 min with 0.4% (w/v) sulforhodamine B (Sigma, St. Louis, MO) dissolved in 1% acetic acid followed by rinsing four times with 1% acetic acid. An A<sub>554</sub> was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and was used as a measure of cell number. The percentage of survival rates of cells exposed to adenovirus vectors were calculated by assuming the survival rate of uninfected cells to be 100%.

**β-Galactosidase Activity following Ad.RSVβgal Infection.** The expression of an adenovirus vector containing the β-galactosidase gene was examined by plating  $2 \times 10^4$  cells in each well of a 96-well plate. The cells were exposed 24 h later to various concentrations of Ad.RSVβgal (0.1–500 pfu/cell) in medium used by each respective cell line except that the serum concentration (if required) was reduced to 2%. After 2-h incubation at 37°C, the serum concentration (if required) was raised to 10%, and then cells were incubated at 37°C for an additional 24 h. Cells were then washed three times with PBS (pH 7.5) and lysed in 50 μl 0.1 M Tris (pH 7.5) containing 0.1% Triton X-100. An aliquot (30 μl) was assayed for β-galactosidase activity using a modified protocol (27). Samples were transferred to each well of 96-well plates and treated with 100 μl 20 mM Tris (pH 7.5) containing 1 mM MgCl<sub>2</sub>, 450 μM β-mercaptoethanol, and 150 μM O-nitrophenyl-β-galactopyranoside. Incubations were performed at 37°C for 20 min, and the reaction was stopped by the addition of 150 μl/well of 1 M Na<sub>2</sub>CO<sub>3</sub>. The

absorbance was determined at 420 nm. An  $A_{420}$  of 1 was defined as 1 unit of enzyme activity.

**Immunoprecipitation of p53 Protein in Cells Infected with AdWtp53.** Cells ( $1 \times 10^6$ ) were plated in 10-cm dishes and infected with AdWtp53 or AdControl for 24 h as described above. Immunoprecipitations were performed using an anti-p53 antibody essentially as described (31). In brief, cells were incubated with 3 ml methionine-free DMEM (Biofluids) containing 5% dialyzed FCS (Biofluids) and 100  $\mu$ Ci/ml [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine mixture (Expres  $^{35}$ S $^{35}$ S-protein labeling mix, 1000 Ci/mmol; New England Nuclear) for 2 h. Cells were washed with ice-cold PBS and solubilized at 4°C in buffer A [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP40, 0.1% sodium deoxycholate (Sigma), 0.5% sodium lauryl sulfate (SDS) (Research Genetics, Huntsville, AL), 1 mM phenylmethyl sulfonylfluoride, 10  $\mu$ g/ml aprotinin, 1.0  $\mu$ g/ml leupeptin, and 1.0  $\mu$ g/ml pepstatin (all protease inhibitors from Boehringer Mannheim)]. Aliquots of 500  $\mu$ l  $^{35}$ S-labeled lysates were incubated with a 1:50 dilution of anti-p53 mAb PAh 1801 (Ab-2; Oncogene Science, Uniondale, NY) at 4°C for 1 h, after which 15  $\mu$ l protein A/G agarose (Oncogene Science) were added, and the incubations were continued for an additional 1 h with rotation. Samples were then centrifuged at  $5000 \times g$  for 5 min, and the pellets were washed successively with buffer A, buffer A containing 1 M NaCl, and finally with buffer A again. SDS gel sample buffer (50  $\mu$ l) was added, and the samples were heated for 5 min at 95°C to elute proteins from the immunoadsorbent. The tubes were centrifuged again at  $5000 \times g$  for 5 min, and 20- $\mu$ l aliquots of protein samples were subjected to SDS-PAGE. Gels were then dried and exposed to X-ray film as described previously (31).

**Western Blot Analysis of p53, WAF1/Cip1, and mdm2 Proteins in Cells Infected with AdWtp53.** Cells ( $0.5 \times 10^6$ ) were plated in 6-cm dishes and incubated with AdWtp53 or AdControl for 24 h as described above. Cells were then washed three times with ice-cold PBS, scraped and resuspended in 1 ml 1X SDS-PAGE buffer (62 mM Tris, pH 6.8, 2 mM EDTA, 15% sucrose, 10% glycerol, 3% SDS, and 0.7 M 2-mercaptoethanol), and boiled for 10 min. Equal amounts (15 or 50  $\mu$ g) of denatured protein were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose filters (29). Filters were blocked with Tris-buffered saline containing 5% dried milk and 0.1% Tween 20 (Sigma). Blots were probed with 4  $\mu$ g/ml Ab-2 and Ab-6 for p53, 4  $\mu$ g/ml EA 10 for WAF1/Cip1, 3  $\mu$ g/ml IF2 for mdm2, or 3  $\mu$ g/ml actin (Ab-1) antibody. All antibodies were obtained from Oncogene Science. After incubation with the primary antibodies, the blots were washed with Tris-buffered saline containing 0.1% Tween 20, incubated with horse radish peroxidase conjugated to secondary antibody, and specific complexes were detected by the enhanced chemiluminescence technique according to manufacturer's directions (New England Nuclear).

**Northern Blot Analysis of WAF1/Cip1 and mdm2 in Cells Infected with AdWtp53.** Cells ( $2 \times 10^6$ ) were plated in 15-cm dishes and incubated with 10 pfu/cell of adenoviral vectors. After incubation for 24 h at 37°C, RNA was extracted by rinsing cells three times with cold PBS and dissolving the cells in a 2-ml solution of guanidine isothiocyanate. RNA was purified by centrifugation over a 5.7 M cesium chloride cushion

(29), fractionated by electrophoresis in agarose gels containing formaldehyde, transferred to Magna NT filters, and cross-linked as described previously (29). Following prehybridization, filters were hybridized using a 2.1-kb fragment of WAF1/Cip1 or an 800-bp fragment from 36B4. Following hybridization, the filters were washed and exposed to X-ray films, and autoradiographs developed as described previously (29).

**Detection of Nucleosomal DNA Fragmentation in Cells Infected with AdWtp53.** For DNA fragmentation studies,  $2 \times 10^6$  cells were plated in 10-cm dishes and incubated with adenovirus vectors (50 pfu/cell) for 24 h. Both adherent and floating cells were collected together and pelleted by centrifugation at  $1800 \times g$  for 5 min (RT-6000B; DuPont, Boston, MA). Cell pellets were rinsed with cold PBS, and low molecular weight DNA was prepared using a modified Hirt extraction method as described previously (22). Briefly, pellets were lysed in 1 ml 10 mM Tris, 10 mM EDTA disodium (pH 7.4; Research Genetics), 0.6% SDS (Research Genetics), and 0.2 mg/ml proteinase K (Boehringer Mannheim). Samples were incubated at 55°C for 5 h, and low molecular weight DNA was prepared using the Hirt extraction method (22) and evaluated by electrophoresis on agarose gel (2%).

The presence of apoptotic cells was also followed by an *in situ* apoptosis detection kit (catalogue number S7110-kit; Oncor, Gaithersburg, MD) according to the manufacturer's instructions.

## RESULTS

**Construction of an AdWtp53.** Homologous recombination between shuttle vector pDK10, containing an expression cassette of human wild-type p53 cDNA and the adenovirus genome cloned in plasmid pJM17, generated an adenovirus clone in which the adenovirus E1 region was replaced by the wild-type p53 cDNA expression cassette. PCR analysis of the purified recombinant adenovirus indicated that it contained p53 cDNA but was devoid of E1a sequences. Fig. 1 is a schematic diagram of AdWtp53. The 5' end of the genome contains the AdWtp53 expression cassette (10.3 mu) followed by the rest of the adenovirus genome. The key elements of the expression cassette of AdWtp53 include the left inverted terminal repeat, adenoviral origin of replication, encapsidation signal, E1a enhancer, cytomegalovirus immediate early promoter, the human wild-type p53 cDNA, and SV40 polyadenylation signal.

**AdWtp53-mediated Synthesis of p53 Protein.** To determine whether AdWtp53 expresses the p53 protein in tumor cells, a lung tumor cell line H-358, which lacks endogenous p53 (2), was exposed to various concentrations of either AdControl or AdWtp53 for 24 h. Following infection, immunoprecipitation of p53 was performed as described in "Materials and Methods." As shown in Fig. 2A, there was no detectable p53 in H358 cells infected with AdControl. In contrast, p53 protein was easily detected by immunoprecipitation in cells infected with 1 pfu/cell of AdWtp53. Furthermore, the amount of immunoprecipitable p53 protein increased with increasing concentrations of AdWtp53 vector.

To investigate adenovirus-mediated p53 expression in breast tumor cells, we exposed several different mammary cell lines (MCF-7, MCF-10, Adr<sup>R</sup> MCF-7, and MDA-MB-231) to AdWtp53 and assessed the synthesis of p53 protein by immu-



Fig. 1 Structure of the recombinant adenoviral vector Ad-WTp53.  $\square$ , adenovirus type 5 genome of 9.24–100 mu. *Stratum*, origin of replication, encapsidation signals, and E1a enhancer derived from adenovirus type 5 (stripped segments); human cytomegalovirus immediate early promoter (left blank segment); human wild-type p53 cDNA (solid segment); and SV40 RNA maturation signal (right blank segment).

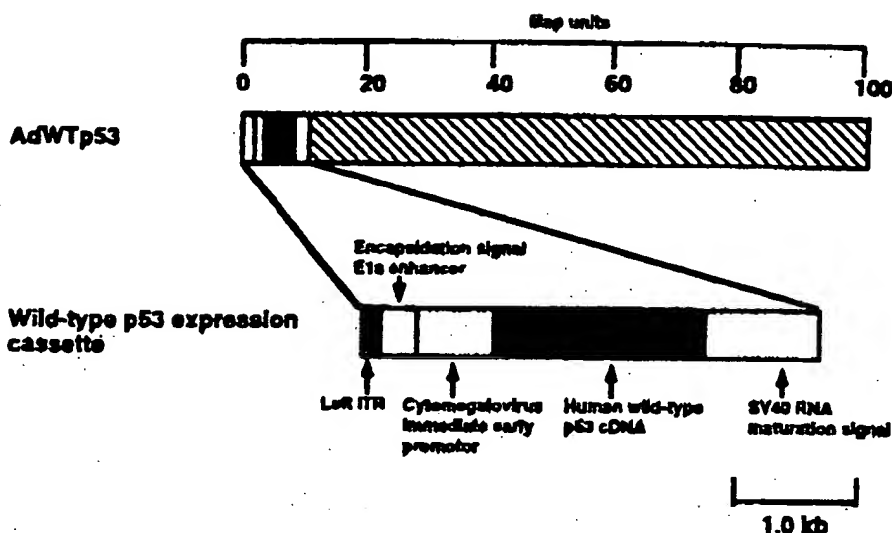


Fig. 2 A, immunoprecipitation of  $^{35}\text{S}$ -labeled human wild-type p53 protein from H-358 cells exposed to various doses of AdWtp53 or AdControl. H-358 cells were exposed to various concentrations of either AdControl or AdWtp53 for 24 h as described in "Materials and Methods." After labeling the cells with [ $^{35}\text{S}$ ]methionine-cysteine, cell lysates were immunoprecipitated using anti-p53 antibody, solubilized protein samples were loaded on 8% SDS-PAGE, and gels were dried and exposed to X-ray film. Left panel, signals of p53 precipitates from H-358 cells exposed to AdControl at 0.1, 1, 10, and 50 pfu/cell and uninfected cells. Right panel, signals of p53 precipitates from H-358 cells exposed to AdWtp53 at 0.1, 1, 10, and 50 pfu/cell. Numbers 0–50 on top of the lanes. Arrow, position of p53 protein on gels. B, immunoprecipitation of  $^{35}\text{S}$ -labeled p53 protein from various cell lines. Various cells (MCF-10, MCF-7, MDA-MB-231, and AdR<sup>+</sup> MCF-7) were exposed to AdWtp53 (50 pfu/cell) or AdControl (50 pfu/cell), and p53 protein was immunoprecipitated as described above. Left panel, results of p53 immunoprecipitation of uninfected cells; middle panel, immunoprecipitation of cells exposed to AdControl; and right panel, results of cells exposed to AdWtp53. Arrow, position of p53 protein on gels.

noprecipitation. As shown in Fig. 2B, MCF-10, MCF-7, AdR<sup>+</sup> MCF-7, and MDA-MB-231 expressed low levels of endogenous p53. However, following exposure of the cells to 10 pfu/cell of AdWtp53, a marked increase in the level of p53 expression was observed in the infected cells. In contrast, infection with AdControl did not result in any increase in p53 expression above that present in uninfected cells. These results were also confirmed by Western blot analysis (see below) and indicate that AdWtp53 can infect both human mammary and lung cells. Moreover, infection with AdWtp53 resulted in high levels of p53 expression in these cells.

**Effect of AdWtp53 on Cell Growth.** We investigated the effect of high-level wild-type p53 expression on the growth of cells having different p53 status. For these studies we used H-358 lung cancer cells, which are devoid of p53 (p53 null; Ref. 2), MDA-MB-231 human breast cancer cells, which express mutant p53 (32), and MCF-7 human breast cancer cells, which express wild-type p53 (16, 32). Each cell line was exposed to 10 pfu/cell of either AdWtp53 or AdControl and harvested daily for cell counts. As shown in Fig. 3, A and B, infection of H-358 and MDA-MB-231 cells with AdWtp53 completely inhibited cell growth over the 4-day period examined. In both of these cell lines, the cell number was reduced by day 4 to levels less than one-half of that present at time 0. In contrast, MCF-7 cells continued to proliferate although at a slower rate than control cells (Fig. 3C). As a control for these experiments, we show that AdControl virus had very little effect on the growth of these cells (Fig. 3).

These results suggested that infection by AdWtp53 had a more profound growth inhibitory effect on cells that were either deficient in p53 or expressed a mutant p53 than on cells that expressed wild-type p53. To confirm these observations, we investigated the effects of AdWtp53 on cancer cells that lacked p53 expression (H-358 and MDA-MB-157; Refs. 2 and 32), cancer cells that expressed endogenous mutant p53 (MDA-MB-231 and MDA-MB-453; Refs. 32 and 33), cancer cells that

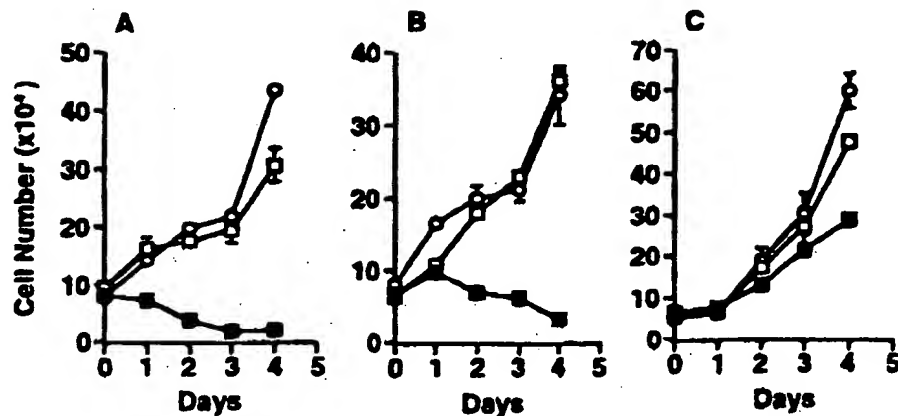


Fig. 3 Effect of AdWtp53 and AdControl on cell growth. Cells ( $5 \times 10^4$ ) were plated in triplicate on 6-well plates, exposed to AdWtp53 (10 pfu/cell) or AdControl (10 pfu/cell), and cell number counted on each day. Shown are cell number of: (A) H-358 cells: uninfected ( $\circ$ ), exposed to AdWtp53 ( $\blacksquare$ ), and exposed to AdControl ( $\square$ ); (B) MDA-MB-231 cells: uninfected ( $\circ$ ), exposed to AdWtp53 ( $\blacksquare$ ), and exposed to AdControl ( $\square$ ); (C) MCF-7 cells: uninfected ( $\circ$ ), exposed to AdWtp53 ( $\blacksquare$ ), and exposed to AdControl ( $\square$ ). Values shown are mean  $\pm$  SE.

Table 1 Summary of the endogenous p53 status of the various cell lines,  $IC_{50}$  values of AdWtp53 in each cell line,  $\beta$ -gal activity in these cells after infecting with Ad.RSV $\beta$ gal vector, and relative induction of WAF1/Cip1 protein expression following infection with AdWtp53

Cell line	Endogenous p53 status <sup>a</sup>	$IC_{50}$ AdWtp53 <sup>b</sup>	$\beta$ -gal Activity <sup>c</sup>	Fold induction WAF1/Cip1 <sup>d</sup>
H-358	Null	0.17	0.75	105
MDA-MB-157	Null	0.30	0.54	83
MDA-MB-231	Mutant	0.4	0.66	154
MDA-MB-453	Mutant	0.70	0.58	71
MCF-7	Wild type	30	0.30	2.3
184B5	Wild type	4.5	ND	2.4
MCF-10	Wild type	5.5	ND	7.5
NMECs	Wild type	100	0.731	1.2

<sup>a</sup> The status of endogenous p53 in each cell line is from Refs. 2, 16, 29, 32, and 33, and J. Gudas *et al.* unpublished data as described in the text.

<sup>b</sup> Values are estimated from the cell killing experiments described in the text.

<sup>c</sup>  $\beta$ -galactosidase activity in each cell line was measured after infecting cells with 20 pfu/cell as described in "Materials and Methods."

<sup>d</sup> Fold increase in the WAF1/Cip1 expression represents the AdWtp53-mediated (10 pfu/cell) increase in WAF1/Cip1 protein expression over the uninfected cells.

expressed wild-type p53 (MCF-7) and immortalized, and normal mammary epithelial cells that expressed wild-type p53 (MCF-10, 184B5, and NMECs)<sup>a</sup> (29). In these experiments each cell line was exposed to increasing concentrations of AdWtp53 for 7 days. As shown in Table 1, cells that are null for expression of p53 were the most sensitive to the inhibitory effect of AdWtp53 ( $IC_{50}$ s for H-358 and MDA-MB-157 cells were 0.17 and 0.32 pfu/cell, respectively). Cells that express a mutant p53

protein were only slightly less sensitive to the growth inhibitory effects of AdWtp53 ( $IC_{50}$ s for MDA-MB-231 and MDA-MB-453 were 0.4 and 0.7 pfu/cell, respectively). In contrast, immortalized or normal cells that expressed wild-type p53 were the most resistant to the cytotoxic effects of AdWtp53, with NMECs being the most resistant ( $IC_{50}$ s for 184B5, MCF-10, MCF-7, and NMEC were 4.5, 5.5, 30, and 100 pfu/cell, respectively; Table 1). Although some of the cytotoxic effects of AdWtp53, particularly at high multiplicity of infection ( $>100$  pfu/cell), could be due to the nonspecific effects of the recombinant adenovirus, in general, it appears that cells that express wild-type p53 were 5–250 times more resistant to the AdWtp53-mediated inhibitory effect on cell growth when compared with cells expressing no p53 or mutant p53.

**Ad.RSV $\beta$ -gal-mediated  $\beta$ -Galactosidase Activity.** Since differences in the sensitivity of various cell lines to AdWtp53 could result from either reduced uptake and/or decreased transgene expression, the expression of an adenovirus vector containing the marker gene  $\beta$ -galactosidase was examined in these cells. Cells were exposed to different concentrations of Ad.RSV $\beta$ -gal for 24 h, and  $\beta$ -galactosidase activity was measured as described in "Materials and Methods." In all cell lines, expression of  $\beta$ -galactosidase was linear in the range of  $\sim 100$  pfu/cell of Ad.RSV $\beta$ -gal (data not shown). Following infection of each cell line at 20 pfu/cell, the enzyme activity in each cell line was in the range of 0.3–0.75 units (Table 1). Moreover, as shown below (Fig. 4), each of the cell lines expressed high amounts of p53 when these cells were exposed to AdWtp53. Therefore, the differences in the sensitivity of killing effects of AdWtp53 cannot be explained by alteration in viral uptake and/or differential expression of the transgene.

**Effect of AdWtp53 on WAF1/Cip1 and mdm2 Protein Expression.** To further investigate the molecular mechanisms underlying the cytotoxicity of AdWtp53, we examined the expression of two cellular proteins that could play a role in mediating the inhibitory effects of p53. These included WAF1/Cip1, a gene which is induced in cells and inhibits cyclin kinase

<sup>a</sup> J. Gudas *et al.*, unpublished data.

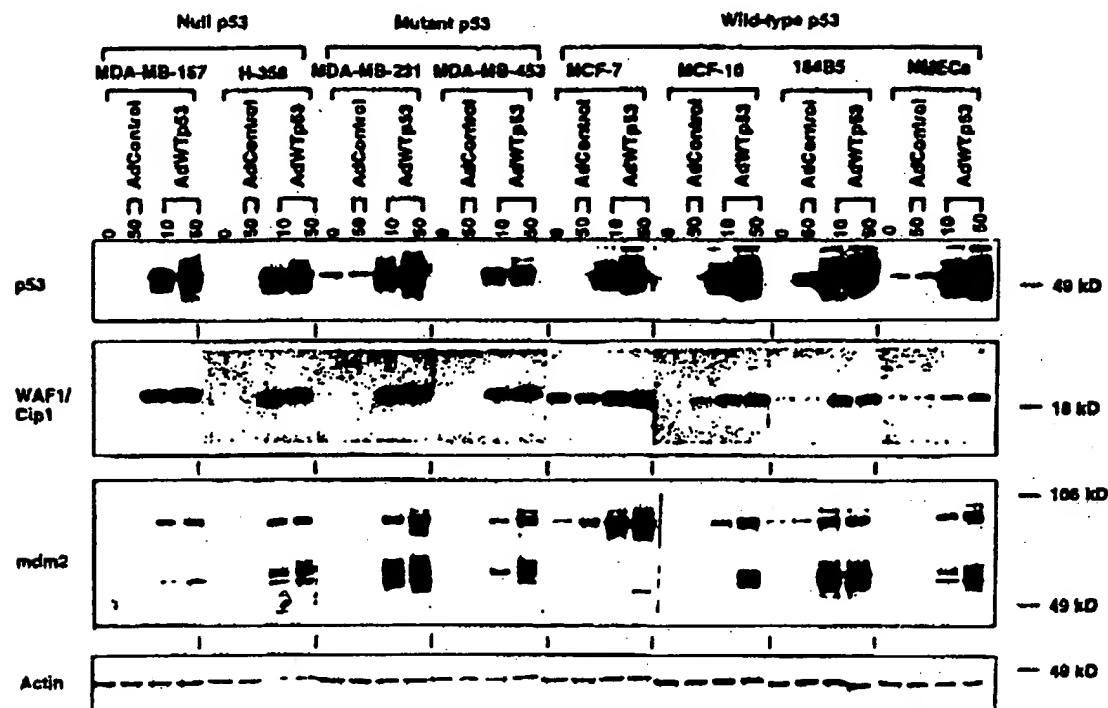


Fig. 4. Western blot analysis of p53, WAF1/Cip1, mdm2, and actin proteins in breast cancer cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-453, and MCF-7), a lung cancer cell line (H-358), immortalized mammary cells (MCF10 and 184B5), and NMECs. Cells ( $0.5 \times 10^7$ ) were plated in 6-cm dishes and infected with either 10 or 50 pfu/cell of AdWTP53 or 50 pfu/cell of AdControl for 24 h. Cells were harvested and resuspended in 1 ml 1X SDS-PAGE buffer, and 15  $\mu$ g protein were separated in an 8% SDS-polyacrylamide gel and electroblotted onto nitrocellulose, and the membranes reacted with antibodies corresponding to p53, WAF1/Cip1, mdm2, and actin. Protein bands were detected by autoradiography of X-ray film. Top of the panel, type of each cell line used. Numbers 10 or 50 on top of the lanes, amount of AdControl or AdWTP53 (pfu/cell). Left side of the panel, antibodies used for detecting proteins. Right side of the panel, protein molecular weight markers.

(34–36), and *mdm2*, another *p53*-inducible gene that can bind *p53* and modulate its function (37).

As shown in Fig. 4, Western blot analyses demonstrated that low levels of endogenous *p53* could be detected in all cell lines examined except MDA-MB-157 and H-358. However, the level of *p53* increased substantially (at least 10-fold) in each cell line following AdWTP53 infection (10 or 50 pfu/cell). In contrast, the amount of *p53* increased little, if at all, above the endogenous *p53* protein level in cells exposed to 50 pfu/cell of AdControl. Because cells exposed to either AdControl or AdWTP53 expressed similar levels of actin protein (Fig. 4), increased levels of *p53* following AdWTP53 infection cannot be due to loading of different amounts of proteins or other non-specific mechanisms.

We also examined the induction of WAF1/Cip1 expression following AdWTP53 infection. As shown in Fig. 4, there was little or no detectable basal level of WAF1/Cip1 in cells that did not express endogenous wild-type *p53* (MDA-MB-157 and H-358), or in cells that expressed a mutant *p53* (MDA-MB-231 and MDA-MB-453), basal levels of WAF1/Cip1 were readily detected in cells that expressed endogenous wild-type *p53* (MCF-7, MCF-10, 184B5, and NMECs). Although exposure of cells to 50 pfu/cell of AdControl did not affect the basal level of WAF1/Cip1 in any of these cells, exposure to 10 or 50 pfu/cell

of AdWTP53 resulted in a marked increase in WAF1/Cip1 expression in all of the cell line. However, cells expressing endogenous mutant *p53* or null for *p53* appeared to induce higher levels (fold-induction) of WAF1/Cip1 protein as compared to cells expressing wild-type *p53* (Fig. 4 and Table 1).

*mdm2* protein levels were also determined before and after AdWTP53 infection in each cell line. Basal levels of *mdm2* protein were not detectable in cells that were null for *p53* or contained mutant *p53*. Endogenous *mdm2* protein bands of approximately 90 kDa and 57 kDa<sup>1</sup> (37) were readily detected in all cells expressing wild-type *p53*, and no difference in the levels of either *mdm2* proteins were observed following infection of cells with the AdControl vector. In contrast, following exposure to AdWTP53 at 10 or 50 pfu/cell, there was a marked increase in the levels of high and low molecular weight *mdm2* proteins in all cell lines examined except MCF-7 cells in which AdWTP53-mediated expression of the 57-kDa protein was minimal.

**Effect of AdWTP53 on WAF1/Cip1 mRNA Expression.** Since *p53* is a DNA-binding transcription factor (38), we determined whether AdWTP53-mediated induction of WAF1/Cip1 protein was regulated at the level of RNA. The expression of WAF1/Cip1 mRNA was assessed by Northern blot analysis following infection of cells with either AdControl or AdWTP53.

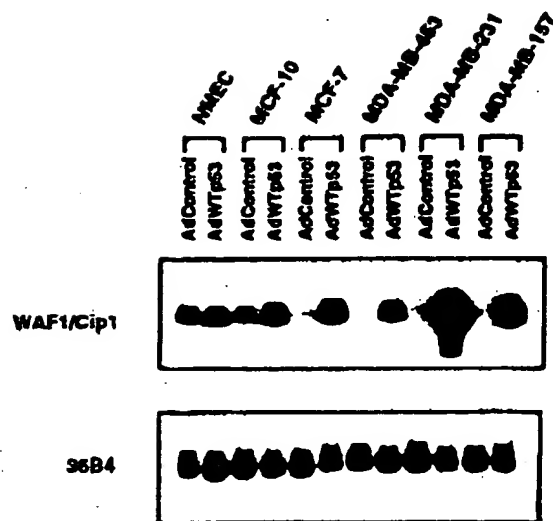


Fig. 5 Northern blot analysis of p53 mRNA in cells exposed to AdWTP53. Cells ( $2 \times 10^6$ ) were plated and 24 h later infected with AdWTP53 or AdControl (10 pfu/cell) for 24 h, and RNA was prepared and subjected to Northern blot analysis. After transferring RNA to Magna NT membranes, blots were either probed with a p53 or 36B4 cDNA probe. Results of autoradiograms obtained from different cells shown on top of the lanes, exposed to either AdControl or AdWTP53.

As shown in Fig. 5, cells devoid of WTP53 (MDA-MB-157) and cells expressing mutant p53 (MDA-MB-453 and MDA-MB-231) had very low levels of WAF1/Cip1 mRNA after infection with AdControl. NMECs, MCF-10 cells, and MCF-7 cells all contained endogenous wild-type p53 and expressed varying levels of WAF1/Cip1 mRNA expression following infection with AdControl. Following infection of AdWTP53, the WAF1/Cip1 mRNA levels in all cell lines increased. There was a 7.4-fold increase in MDA-MB-453, a 21-fold increase in MDA-MB-231, an 8.2-fold increase in MDA-MB-157 cells, a 6-fold increase in MCF-7 cells, a 2-fold increase in MCF-10 cells, and a 1.2-fold increase in NMECs. As a control for these experiments, we show that the level of a control mRNA (36B4) was similar in cells infected with either AdControl or AdWTP53. Thus, the induction of WAF1/Cip1 proteins in cells after infection with AdWTP53 appears to be mediated by an increase in WAF1/Cip1 mRNA, although the possibility of a posttranscriptional regulation of p53 expression cannot be ruled out.

**AdWTP53-mediated Apoptosis.** To investigate whether the mechanisms of AdWTP53-mediated inhibition of cell growth involved programmed cell death (apoptosis), the effect of AdWTP53 on nucleosomal DNA fragmentation was examined after infection of MDA-MB-231 cells (which express endogenous mutant p53), H-358 cells (which are null for p53), and in MCF-7 and NMECs (both of which express endogenous wild-type p53). As shown in Fig. 6, 24 h after exposure of MDA-MB-231 cells to 50 pfu/cell of AdWTP53, several lower molecular weight DNA bands (DNA laddering of approximately 145 bp) in the range of 145-1050 bp were observed, which are characteristic of cells undergoing apoptosis. Similar results were observed following AdWTP53 infection of H-358 cells (data not

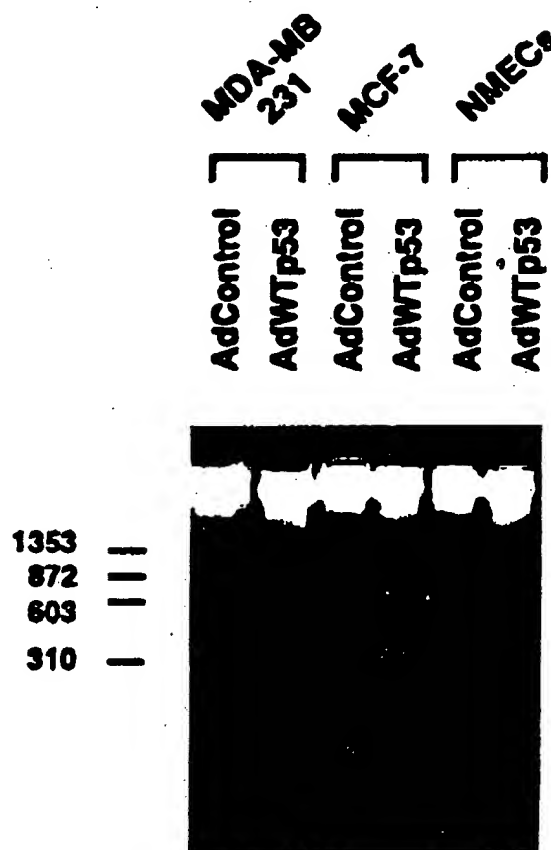


Fig. 6 Nucleosomal DNA fragmentation in AdWTP53-infected MDA-MB-231, MCF-7, and NMECs. Cells ( $2 \times 10^6$ ) were plated in 10-cm dishes and exposed to either AdControl or AdWTP53. One day after infection, cells were collected and incubated with a lysis buffer, and low molecular weight DNA was prepared and subjected to an agarose gel electrophoresis. Results shown are the DNA pattern observed in various cell lines (top of the lane) infected with 50 pfu/cell of either AdControl or AdWTP53. Numbers on the left, position of molecular weight markers (bp).

shown). In contrast, exposure of MDA-MB-231 cells to AdControl or mock infection of these cells produced no detectable DNA laddering (Fig. 6). Exposure of MCF-7 cells or NMECs to AdWTP53 at 50 pfu/cell or MCF-7 cells to 1000 pfu/cell also did not show any DNA laddering (data not shown). Similarly, when MDA-MB-231, H-358, and MCF-7 cells were infected with AdWTP53 for 24 h, followed by the detection of apoptotic cells by an *in situ* apoptosis detection deoxynucleotidyl transferase end labeling, while MDA-MB-231 and H-358 cells appeared to show a significant number of fluorescent apoptotic cells, no apoptotic cells were present in MCF-7 cell population (data not shown). These results indicate that tumor cells null for p53 or expressing an endogenous mutant p53 undergo apoptosis following exposure to AdWTP53, whereas tumor cells or normal cells expressing wild-type p53 are resistant to apoptosis.

## DISCUSSION

We have constructed an adenovirus vector expressing a human wild-type p53 protein (AdWTP53) that produces high levels of p53 protein when introduced into normal and malignant human mammary epithelial cells. The rapid induction of wild-type p53 protein following infection of cells with AdWTP53 provided an opportunity to study the biological effects of p53 in cells which differ in their expression of the endogenous p53 gene.

Tumor cells expressing endogenous mutant p53 or devoid of p53 expression were more sensitive to AdWTP53-mediated cytotoxicity when compared with tumor or normal cells expressing endogenous wild-type p53. Moreover, overexpression of wild-type p53 induced programmed cell death (apoptosis) of tumor cells devoid of wild-type p53 or expressing endogenous mutant p53, but not in tumor or normal cells expressing wild-type p53. There are several possible mechanisms by which high expression of wild-type p53 results in apoptosis in tumor cells devoid of p53 or expressing mutant p53, but not in tumor or normal cells expressing wild-type p53. For example, cell killing could be dependent on the amount of p53 produced in different cells, stability of p53 protein in different cells, localization of p53 within the cell and the ability (inability) of p53 protein to interact with other cellular factors, and downstream signal transduction pathway. Moreover, as previously suggested (39) a translational modification of p53 may play a role in p53-induced apoptosis.

To understand the differential effects of wild-type p53 overexpression in cells with a different intrinsic p53 status, we investigated the expression of two proteins that are known to be regulated by wild-type p53, WAF1/Cip1 and mdm2. AdWTP53-mediated cytotoxic effects appeared to be associated with the high induction of WAF1/Cip1. The WAF1/Cip1 gene has been shown to bind to cellular cyclin-dependent kinases and thereby inhibit their function (34-36). This inhibition is manifested in turn by a decrease in the level of phosphorylation of the Rb protein (40). Preliminary work in our laboratory has suggested that AdWTP53-mediated p53 protein induction in cells devoid of p53 or expressing mutant p53 is associated with dephosphorylation of the Rb protein. On the other hand, in cells expressing wild-type p53, the phosphorylated form of the Rb protein was still present after infection with AdWTP53 (data not shown). Thus, marked induction of WAF1/Cip1 expression after infection with AdWTP53 in turn is associated with an inhibition of the phosphorylation of the Rb protein.

Exposure of cells to DNA-damaging agents such as radiation resulted in apoptosis in normal thymocytes or other cells expressing wild-type p53 (6, 7, 41), while thymocytes and other cancer cells null for p53 or containing endogenous mutant p53 were resistant to radiation-mediated cell death (41, 42). The effects of radiation-induced p53 were suggested to be mediated by WAF1/Cip1 induction (41). In the current study we observed that high expression of p53 protein by AdWTP53 was associated with a marked induction of WAF1/Cip1 RNA and protein. Although both radiation and AdWTP53 can induce WAF1/Cip1 protein, whether WAF1/Cip1 is directly responsible for apoptosis is not known. The possibility of a signal transduction agent other than WAF1/Cip1 that mediates the induction of apoptosis

in cells by p53 is also possible. Our future experiments will be directed toward understanding the role of WAF1/Cip1 and other signal transduction agents in AdWTP53-mediated apoptosis.

The presence of mutated p53 is widespread in different human cancers. Thus, reconstituting tumor suppressor p53 gene expression by adenovirus vectors is an attractive strategy for cancer gene therapy. Since adenovirus enters human epithelial cells with an efficient low pH endosome-mediated endocytosis (19, 21), tumors of mammary epithelial origin will be especially amenable to treatment by AdWTP53. Previous studies by Liu *et al.* (43) have shown that adenovirus-mediated expression of p53 could cause killing of cells derived from head and neck tumors and of lung cancer cells in the presence of cis-platinum (44). As shown here, normal mammary epithelial cells are resistant to apoptosis by AdWTP53, while the tumor cells null for p53 or expressing mutant p53 readily undergo apoptosis. Thus, there is a specificity to AdWTP53-mediated killing of tumor cells, lending further support to these vectors for gene therapy of cancer.

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## REFERENCES

1. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. C., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. Mutations in the p53 gene occur in diverse human tumor types. *Nature (Lond.)*, 342: 705-708, 1989.
2. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. *Science (Washington DC)*, 246: 441-444, 1989.
3. Srivastava, S., Zou, Z., Pirolo, K., Blattner, W., and Chang, E. H. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature (Lond.)*, 344: 747-749, 1990.
4. Chen, P., Chen, Y., Bookstein, R., and Lee, W. Genetic mechanisms of tumor suppression by the human p53 gene. *Science (Washington DC)*, 250: 1576-1579, 1991.
5. Gottesman, M. M. Report of a meeting: molecular basis of cancer therapy. *J. Natl. Cancer Inst.*, 86: 1277-1285, 1994.
6. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature (Lond.)*, 362: 849-852, 1993.
7. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature (Lond.)*, 362: 847-849, 1993.
8. Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G<sub>1</sub> arrest. *Cell*, 76: 1013-1023, 1994.
9. Nelson, W. G., and Kastan, M. B. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol. Cell. Biol.*, 14: 1815-1823, 1994.
10. Michalovitz, D., Halevy, O., and Oren, M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell*, 62: 671-680, 1990.

11. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature (Lond.)*, 352: 345-347, 1991.
12. Chiou, S., Rao, L., and White, E. Bcl-2 blocks p53-dependent apoptosis. *Mol. Cell. Biol.*, 14: 2556-2563, 1994.
13. Donchower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature (Lond.)*, 356: 215-221, 1992.
14. Shaw, P., Bowey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. USA*, 89: 4495-4499, 1992.
15. Lowe, S. W., Jacks, T., Housman, D. E., and Riley, H. E. Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. *Proc. Natl. Acad. Sci. USA*, 91: 2026-2031, 1994.
16. Cassey, G., Lo-Hsueh, M., Lopez, M. E., Vogelstein, B., and Stanbridge, E. J. Growth suppression of human breast cancer cells by the introduction of a wild-type p53 gene. *Oncogene*, 6: 1817-1811, 1991.
17. Ginsberg, H. S. Adenoviruses. In: R. Dulbecco and H. S. Ginsberg (eds.), *Virology*, pp. 147-160. Philadelphia: J. B. Lippincott Co., 1988.
18. Graham, F. L., and Prevec, L. Manipulation of adenovirus vectors. In: E. J. Murray (ed.), *Gene Transfer and Expression Protocols*, pp. 109-128. Clifton, NJ: Humana Press, 1991.
19. Seth, P., Fitzgerald, D., Ginsberg, H., Willingham, M., and Pastan, I. Pathway of adenovirus entry into cells. In: R. Crowell and K. Longberg-Holm (eds.), *Virus Attachment and Entry into Cells*, pp. 191-195. Washington, DC: American Society for Microbiology, 1986.
20. Seth, P., Rosenfeld, M., Higginbotham, J., and Crystal, R. G. Mechanism of enhancement of DNA expression consequent to coinfection of a replication-deficient adenovirus and unmodified plasmid DNA. *J. Virol.*, 68: 933-940, 1994.
21. Jones, N., and Shenk, T. Isolation of adenovirus type 5 burst range deletion mutants defective for transformation of rat embryo cells. *Cell*, 17: 683-689, 1979.
22. Rosenfeld, M. A., Chu, C., Seth, P., Daniel, C., Banks, T., Yoneyama, K., Yoshimura, K., and Crystal, R. G. Gene transfer to freshly isolated human respiratory epithelial cells *in vitro* using a replication-deficient adenovirus containing the human cystic fibrosis transmembrane conductance regulator cDNA. *Hum. Gene Ther.*, 5: 331-342, 1994.
23. Zakut-Houri, R., Bienz-Tadmor, B., Givol, D., and Oren, M. Human p53 cellular tumor antigen: cDNA sequence and expression in COS cells. *EMBO J.*, 4: 1251-1255, 1985.
24. McGrory, W. J., Bautista, D. S., and Graham, F. L. A simple technique for the rescue of early region 1 mutation into infectious human adenovirus type 5. *Virology*, 163: 614-617, 1988.
25. Gilardi, P., Courtney, M., Pavirani, A., and Perricaudet, M. Expression of human alpha1-antitrypsin using a recombinant adenovirus vector. *FEBS Lett.*, 267: 60-62, 1990.
26. Katayose, D., Ohe, M., Yamachi, K., Ogata, M., Shirao, K., Fujita, H., Shibahara, S., and Takishima, T. Increased expression of PDGF A- and B-chain genes in rat lungs with hypoxic pulmonary hypertension. *Am. J. Physiol.*, 264: L100-L106, 1993.
27. Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M., and Briand, P. J. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.*, 90: 626-630, 1992.
28. Fairchild, C. R., Ivy, S. P., Kao-Shan, C., Whang-Peng, J., Rosen, N., Israel, M. A., Melera, P. W., Cowan, K. H., and Goldsmith, M. E. Isolation of amplified and overexpressed DNA sequences from adriamycin-resistant human breast cancer cells. *Cancer Res.*, 47: 5141-5148, 1987.
29. Gudaz, J. M., Oka, M., Diella, F., Trepel, J., and Cowan, K. H. Expression of wild-type p53 during the cell cycle in normal human mammary epithelial cells. *Cell Growth & Differ.*, 5: 295-304, 1994.
30. Skehan, P., Storeng, R., Scudiero, D., Munks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer drug screening. *J. Natl. Cancer Inst.*, 82: 1107-1112, 1990.
31. Seth, P., Fitzgerald, D., Ginsberg, H., Willingham, M., and Pastan, I. Evidence that the penton base of adenovirus is involved in potentiation of toxicity of pseudomonas aeruginosa conjugated to epidermal growth factor. *Mol. Cell. Biol.*, 4: 1528-1533, 1984.
32. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. Genetic and immunohistochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene*, 5: 893-899, 1990.
33. Halder, S., Negrini, M., Monne, M., Sabbioni, S., and Croce, C. M. Down-regulation of bcl-2 by p53 in breast cancer cells. *Cancer Res.*, 54: 2095-2097, 1994.
34. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kohayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinase. *Nature (Lond.)*, 366: 701-704, 1993.
35. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell*, 75: 817-825, 1993.
36. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting Cipl is a potential inhibitor of G1 cyclin-dependent kinases. *Cell*, 75: 805-816, 1993.
37. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. L. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, 69: 1237-1245, 1992.
38. Ullrich, S. J., Anderson, C. W., Mercer, W. E., and Appella, E. The p53 tumor suppressor protein, a modulator of cell proliferation. *J. Biol. Chem.*, 267: 15259-15262, 1992.
39. Caclies, C., Helmsberg, A., and Karin, M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature (Lond.)*, 370: 220-223, 1994.
40. Ewen, M. E. The cell cycle and the retinoblastoma protein family. *Cancer Metastasis Rev.*, 13: 45-66, 1994.
41. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W., and Vogelstein, B. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.*, 54: 1169-1174, 1994.
42. McIlwraith, A. J., Vasey, P. A., Ross, G. M., and Brown, R. Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity. *Cancer Res.*, 54: 3718-3722, 1994.
43. Liu, T., Zhang, W., Taylor, D. L., Roth, J. A., Goepfert, H., and Clayman, G. L. Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via recombinant adenovirus. *Cancer Res.*, 54: 3662-3667, 1994.
44. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., Zhang, W., Owen-Schaub, L. B., and Roth, J. A. Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type p53 gene. *Cancer Res.*, 54: 2287-2291, 1994.

# Development and Characterization of Recombinant Adenoviruses Encoding Human p53 for Gene Therapy of Cancer

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## ABSTRACT

We have constructed recombinant human adenoviruses that express wild-type human p53 under the control of either the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early gene promoter. Each construct replaces the Ad 5 E1a and E1b coding sequences necessary for viral replication with the p53 cDNA and MLP or CMV promoter. These p53/Ad recombinants are able to express p53 protein in a dose-dependent manner in infected human cancer cells. Tumor suppressor activity of the expressed p53 protein was assayed by several methods. [<sup>3</sup>H]Thymidine incorporation assays showed that the recombinant adenoviruses were capable of inhibiting DNA synthesis in a p53-specific, dose-dependent fashion. *Ex vivo* treatment of Saos-2 tumor cells, followed by injection of the treated cells into nude mice, led to complete tumor suppression using the MLP/p53 recombinant. Following a single injection of CMV/p53 recombinant adenovirus into the peritumoral space surrounding an *in vivo* established tumor derived from a human small cell lung carcinoma cell line (NIH-H69), we were able to detect p53 mRNA in the tumors at 2 and 7 days post-injection. Continued treatment of established H69 tumors with MLP/p53 recombinant led to reduced tumor growth and increased survival time compared to control treated animals. These results indicate that recombinant adenoviruses expressing wild-type p53 may be useful vectors for gene therapy of human cancer.



## OVERVIEW SUMMARY

Introduction of the p53 tumor suppressor gene into tumors bearing p53 mutations can inhibit cellular proliferation and tumorigenicity. Wills *et al.* describe replication-deficient recombinant adenoviruses directing expression of human p53 both *in vitro* and *in vivo*. They show that adenovirus-mediated expression of wild-type p53 in p53 altered tumors can suppress proliferation and inhibit tumorigenicity in *ex vivo* and *in vivo* cancer models.

## INTRODUCTION

**M**UTATION OF THE P53 GENE is the most common genetic alteration in human cancers (Bartek *et al.*, 1991; Holl-

stein *et al.*, 1991). In its proposed role as a "guardian of the genome" (Lane, 1992), the p53 gene product functions as a transcriptional activator of other genes which inhibit cell cycle progression from G<sub>1</sub> to S phase in normal cells. Its levels rise and accumulate in response to DNA damage, leading either to G<sub>1</sub> arrest and repair, terminal differentiation, or, if too much damage has occurred, apoptosis (Kuerbitz *et al.*, 1992; Lane, 1992). Loss of wild-type p53 function is associated with the uncontrolled growth of many types of human cancers. The reexpression of normal p53 in p53-altered tumor cells has been demonstrated to suppress tumor growth (Chen *et al.*, 1990; Cheng *et al.*, 1992; Takahashi *et al.*, 1992) or induce apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992). Therefore, p53 functions as a tumor suppressor, restoring a nontumorigenic phenotype to tumor cells in which the endogenous p53 gene has been deleted or mutated.



Recent work has shown that human adenoviruses can be used to deliver genes successfully into a variety of cells and tissues (Lemarchand *et al.*, 1992; Rosenfeld *et al.*, 1992; Rich *et al.*, 1993). Recombinant adenoviruses have several advantages over alternative gene delivery systems such as retrovirus (RV) or adeno-associated virus (AAV)-based vectors for the treatment of cancer. These include the ability to produce stable, high-titer virus capable of efficient infection and subsequent gene expression in target cells (for review, see Siegfried, 1993). Because of the advantages of an adenovirus-based delivery system over other systems for the potential gene therapy of cancer, we constructed recombinant adenoviruses encoding wild-type p53 under the control of the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) promoter. We have tested the ability of these constructs to suppress tumor growth both *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Cell lines

Recombinant adenoviruses were grown and propagated in the human embryonal kidney cell line 293 (ATCC CRL 1573) maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). Saos-2 cells were maintained in Kaighn's media supplemented with 15% fetal calf serum. HeLa and Hep 3B cells were maintained in DME medium supplemented with 10% fetal calf serum. All other cell lines were grown in Kaighn's media supplemented with 10% fetal calf serum. Saos-2 cells were kindly provided by Dr. Eric Stanbridge. All other cell lines were obtained from ATCC.

### Construction of recombinant adenoviruses

To construct the Ad5/p53 viruses, a 1.4-kb *Hind* III-*Sma* I fragment containing the full-length cDNA for p53 was isolated from pGEM1-p53-B-T (kindly supplied by Dr. Wen-Hwa Lee) and inserted into the multiple cloning site of the expression vector pSP72 (Promega) using standard cloning procedures (Sambrook *et al.*, 1989). The p53 insert was recovered from this vector following digestion with *Xho* I-*Bgl* II and gel electrophoresis. The p53 coding sequence was then inserted into either pNL3C or pNL3CMV adenovirus gene transfer vectors (kindly provided by Dr. Robert Schneider), which contain the Ad5 5' inverted terminal repeat and viral packaging signals and the E1a enhancer upstream of either the Ad2 major late promoter (MLP) or the human cytomegalovirus immediate early gene promoter (CMV), followed by the tripartite leader cDNA and Ad 5 sequence 3,325-5,525 bp in a pML2 background. These new constructs replace the E1 region (bp 360-3,325) of Ad5 with p53 driven by either the Ad2 MLP (A/M/53) or the human CMV promoter (A/C/53), both followed by the tripartite leader cDNA (see Fig. 1). The p53 inserts use the remaining downstream E1b polyadenylation site. Additional MLP- and CMV-driven p53 recombinants (A/M/N/53, A/C/N/53) were generated which had a further 705-nucleotide deletion of Ad 5 sequence to remove the protein IX (pIX) coding region. As a control, a recombinant adenovirus was generated from the parental pNL3C plasmid without a p53 insert (A/M). A second

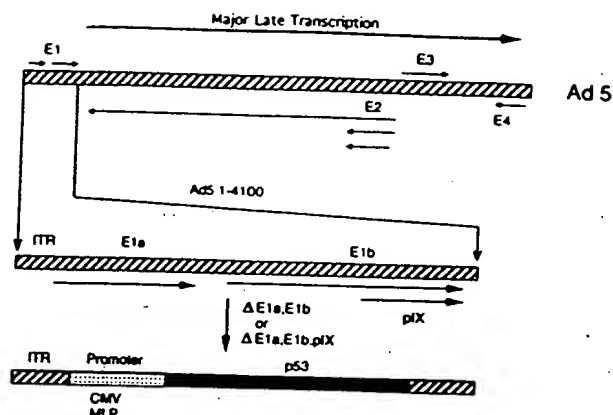


FIG. 1. Schematic of recombinant p53/adenovirus constructs. The p53 recombinants are based on Ad 5 and have had the E1 region of nucleotides 360-3,325 replaced with a 1.4-kb full-length p53 cDNA driven by the Ad 2 MLP (A/M/53) or human CMV (A/C/53) promoters followed by the Ad 2 tripartite leader cDNA. The control virus A/M has the same Ad 5 deletions as the A/M/53 virus, but lacks the 1.4-kb p53 cDNA insert. The remaining E1b sequence (705 nucleotides) have been deleted to create the protein IX-deleted constructs A/M/N/53 and A/C/N/53. These constructs also have a 1.9-kb *Xba* I deletion within adenovirus type 5 region E3.

control (kindly provided by Dr. Robert Schneider) consisted of a recombinant adenovirus encoding the  $\beta$ -galactosidase ( $\beta$ -Gal) gene under the control of the CMV promoter (A/C/ $\beta$ -Gal). The plasmids were linearized with either *Nru* I or *Eco* RI and co-transfected with the large fragment of a *Cla* I-digested Ad 5 *dl309* or *dl327* mutants (Jones and Shenk, 1979; Thimmappaya *et al.*, 1982) using a  $\text{Ca}/\text{PO}_4$  transfection kit (Stratagene). Only the pIX-minus constructs used the *dl327* background which contains a 1.9-kb *Xba* I deletion in the E3 region. Viral plaques were isolated and recombinants identified by both restriction digest analysis and the polymerase chain reaction (PCR) using recombinant-specific primers against the tripartite leader cDNA sequence with downstream p53 cDNA sequence. Recombinant virus was further purified by limiting dilution, and virus particles were purified and titered by standard methods (Graham and van der Erb, 1973; Graham and Prevec, 1991).

### p53 protein detection

Saos-2 or Hep 3B cells ( $5 \times 10^5$ ) were infected with the indicated recombinant adenoviruses for a period of 24 hr at increasing multiplicities of infection (moi) of plaque-forming units of virus/cell. Purified adenovirus, stored in 1% sucrose in phosphate-buffered saline (PBS), is diluted with media to obtain the desired moi and added to plates of cells containing fresh media. After 24 hr, the cells were washed once with PBS and harvested in lysis buffer [50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% NP-40, 50 mM NaF, 5 mM EDTA, 10  $\mu\text{g}/\text{ml}$  aprotinin, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. A Bradford assay (Bio-Rad Protein Assay kit) was used to measure cellular protein concentration, and equal amounts of protein (approximately 30  $\mu\text{g}$ ) were separated



by 10% SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with  $\alpha$ -p53 antibody PAb 1801 (Novocastro) followed by sheep anti-mouse IgG conjugated with horseradish peroxidase. p53 protein was visualized by chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

#### Measurement of DNA synthesis rate

Cells ( $5 \times 10^3$ /well) were plated in 96-well titer plates (Costar) and allowed to attach overnight ( $37^\circ\text{C}$ , 7%  $\text{CO}_2$ ). Cells were then infected for 24 hr with purified recombinant virus particles at moi values ranging from 0.3 to 100, as indicated. Media were changed 24 hr after infection, and incubation was continued for a total of 72 hr. [ $^3\text{H}$ ]Thymidine (Amersham, 1  $\mu\text{Ci}$ /well) was added 18 hr prior to harvest. Cells were harvested on glass fiber filters and levels of incorporated radioactivity were measured in a beta scintillation counter. [ $^3\text{H}$ ]Thymidine incorporation was expressed as the mean % ( $\pm$ SD) of media control and plotted versus the moi.

#### Tumorigenicity in nude mice

Approximately  $2.4 \times 10^6$  Saos-2 cells, plated in T225 flasks, were treated with suspension buffer (1% sucrose in PBS) containing either A/M/N/53- or A/M-purified virus at an moi of 3 or 30. Following an overnight infection, cells were injected subcutaneously into the left and right flanks of BALB/c athymic nude mice (4 mice per group). One flank was injected with the A/M/N/53-treated cells, while the contralateral flank was injected with the control A/M-treated cells, each mouse serving as its own control. Animals receiving bilateral injection of buffer-treated cells served as additional controls. Tumor dimensions (length, width, and height) and body weights were then measured twice per week over an 8-week period. Tumor volumes were estimated for each animal, assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

#### Intratumoral RNA analysis

Female BALB/c athymic nude mice (approximately 5 weeks of age) were injected subcutaneously with  $1 \times 10^7$  H69 small cell lung carcinoma (SCLC) cells in a 200- $\mu\text{l}$  volume in their right flanks. Tumors were then allowed to progress for 32 days. Mice then received peritumoral injections of either A/C/53 or A/C/ $\beta$ -Gal recombinant adenovirus ( $2 \times 10^9$  plaque-forming units (pfu)) into the subcutaneous space beneath the tumor mass. Tumors were excised from the animals 2 and 7 days post adenovirus treatment and rinsed with PBS. Tumor samples were homogenized, and total RNA was isolated using a TriReagent kit (Molecular Research Center, Inc.). Poly(A) RNA was isolated using the PolyAtract mRNA Isolation System (Promega), and approximately 10 ng of sample was used for reverse transcriptase (RT)-PCR determination of recombinant p53 mRNA expression (Wang *et al.*, 1989). Primers were designed to amplify sequence between the adenovirus tripartite leader cDNA and the downstream p53 cDNA, ensuring that only recombinant, and not endogenous p53 would be amplified.

#### p53 gene therapy of established tumors in nude mice

Approximately  $1 \times 10^7$  H69 (SCLC) tumor cells in 200- $\mu\text{l}$  volumes were injected subcutaneously into female BALB/c athymic nude mice. Tumors were allowed to develop for 2 weeks, at which point animals were randomized by tumor size ( $n = 5$ /group). Peritumoral injections of either A/M/N/53 or the control A/M adenovirus ( $2 \times 10^9$  pfu/injection) or buffer alone (1% sucrose in PBS) were administered twice per week for a total of 8 doses/animal per group. Tumor dimensions and body weights were measured twice per week for 7 weeks, and tumor volume was estimated as described previously. Animals were then followed to observe the effect of treatment on mouse survival.

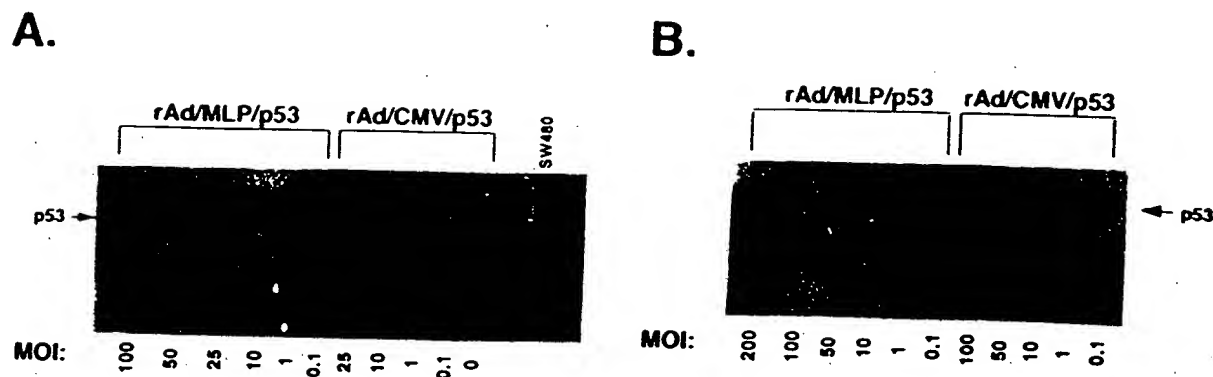
## RESULTS

#### Construction of recombinant p53-adenovirus

p53 adenoviruses were constructed by replacing a portion of the E1a and E1b region of adenovirus type 5 with p53 cDNA under the control of either the Ad2 MLP (A/M/53) or CMV (A/C/53) promoter (schematized in Fig. 1). This E1 substitution severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells that supply Ad 5 E1 gene products *in trans* (Graham *et al.*, 1977). After identification of p53 recombinant adenovirus by both restriction digest and PCR analysis, the entire p53 cDNA sequence from one of the recombinant adenoviruses (A/M/53) was sequenced to verify that it was free of mutations. Following this, purified preparations of the p53 recombinants were used to infect HeLa cells to assay for the presence of phenotypically wild-type adenovirus. HeLa cells, which are nonpermissive for replication of E1-deleted adenovirus, were infected with  $1\text{--}4 \times 10^9$  infectious units of recombinant adenovirus at an moi = 50, cultured for 3 weeks, and observed for the appearance of cytopathic effect (CPE). Using this assay, we were not able to detect recombinant adenovirus replication or wild-type contamination, readily evident by the CPE observed in control cells infected with wild-type adenovirus at a level of sensitivity of approximately 1 in  $10^9$ .

#### p53 protein expression from recombinant adenovirus

To determine if our p53 recombinant adenoviruses expressed p53 protein, we infected tumor cell lines that do not express endogenous p53 protein. The human tumor cell lines Saos-2 (osteosarcoma) and Hep 3B (hepatocellular carcinoma), which contain mutations that result in no expression of p53 protein (Chen *et al.*, 1990; Hsu *et al.*, 1993), were infected for 24 hr with the p53 recombinant adenoviruses A/M/53 or A/C/53 at moi values ranging from 0.1 to 200 pfu/cell. Western analysis of lysates prepared from infected cells demonstrated a dose-dependent p53 protein expression in both cell types (Fig. 2). Both cell lines expressed higher levels of p53 protein following infection with A/C/53 than with A/M/53 (Fig. 2). No p53 protein was detected in noninfected cells. Cells infected with moi values of up to 200 of the control virus A/M also did not show detectable p53 protein (unpublished observation). SW 480 cell



**FIG. 2.** p53 protein expression in tumor cells infected with A/M/53 and A/C/53. A. Saos-2 (osteosarcoma) cells were infected at the indicated moi with either the A/M/53- or A/C/53-purified virus and harvested 24 hr later. The p53 antibody pAb 1801 was used to stain immunoblots of samples loaded at equal total protein concentrations. Equal protein concentrations of SW480 cell extracts, which overexpress mutant p53 protein, were used as a marker for p53 size. The zero (0) under the A/C/53 heading indicates a mock infection containing untreated Saos-2 lysate. B. Hep 3B (hepatocellular carcinoma) cells were infected with the A/M/53 or A/C/53 virus at the indicated moi and analyzed as in A. The arrow indicates the position of the p53 protein.

lysate, which overexpresses mutant p53 protein (Baker *et al.*, 1990), was used as a size marker. Levels of endogenous wild-type p53 are normally quite low, and nearly undetectable by Western analysis of cell extracts (Bartek *et al.*, 1991). It is clear however that wild-type p53 protein levels are easily detectable after infection with either A/M/53 or A/C/53 at the lower moi values (Fig. 2), suggesting that even low doses of p53 recombinant adenoviruses can produce potentially efficacious levels of p53.

#### p53-dependent morphology changes

The reintroduction of wild-type p53 into the p53-negative osteosarcoma cell line, Saos-2, results in a characteristic enlargement and flattening of these normally spindle-shaped cells (Chen *et al.*, 1990). Subconfluent Saos-2 cells ( $1 \times 10^5$  cells/10-cm plate) were infected at an moi of 50 with either the A/C/53 or control A/M virus, and incubated at 37°C for 72 hr until uninfected control plates were confluent. At this point, the expected morphological change was evident in the A/C/53-treated plate (Fig. 3C), but not in uninfected (Fig. 3A) or control virus-infected plates (Fig. 3B). This effect was not a function of cell density because a control plate initially seeded at lower density retained normal morphology at 72 hr when its

confluence approximated that of the A/C/53-treated plate (data not shown). Our previous results had demonstrated a high level of p53 protein expression at a moi of 50 in Saos-2 cells (Fig. 2A), and these results provided evidence that the p53 protein expressed by these recombinant adenoviruses was biologically active.

#### p53 inhibition of cellular DNA synthesis

To test further the activity of the p53 recombinant adenoviruses, we assayed their ability to inhibit proliferation of human tumor cells as measured by the uptake of [ $^3$ H]thymidine. It has previously been shown that introduction of wild-type p53 into cells that do not express endogenous wild-type p53 can arrest the cells at the G<sub>1</sub>/S transition, leading to inhibition of uptake of labeled thymidine into newly synthesized DNA (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990). We infected a variety of p53-deficient tumor cell lines with either A/M/N/53, A/C/N/53 or a non-p53-expressing control recombinant adenovirus (A/M). We observed a strong, dose-dependent inhibition of DNA synthesis by both the A/M/N/53 and A/C/N/53 recombinants in 7 out of the 9 different tumor cell lines tested (Fig. 4). Both constructs were able to inhibit DNA synthesis specifically in these human tumor cells, regardless of whether they ex-



**FIG. 3.** p53-dependent Saos-2 morphology change. Subconfluent ( $1 \times 10^5$  cells/10-cm plate) Saos-2 cells were either uninfected (A), infected at a moi = 50 with the control A/M virus (B), or the A/C/53 virus (C). The cells were photographed 72 hr post-infection.

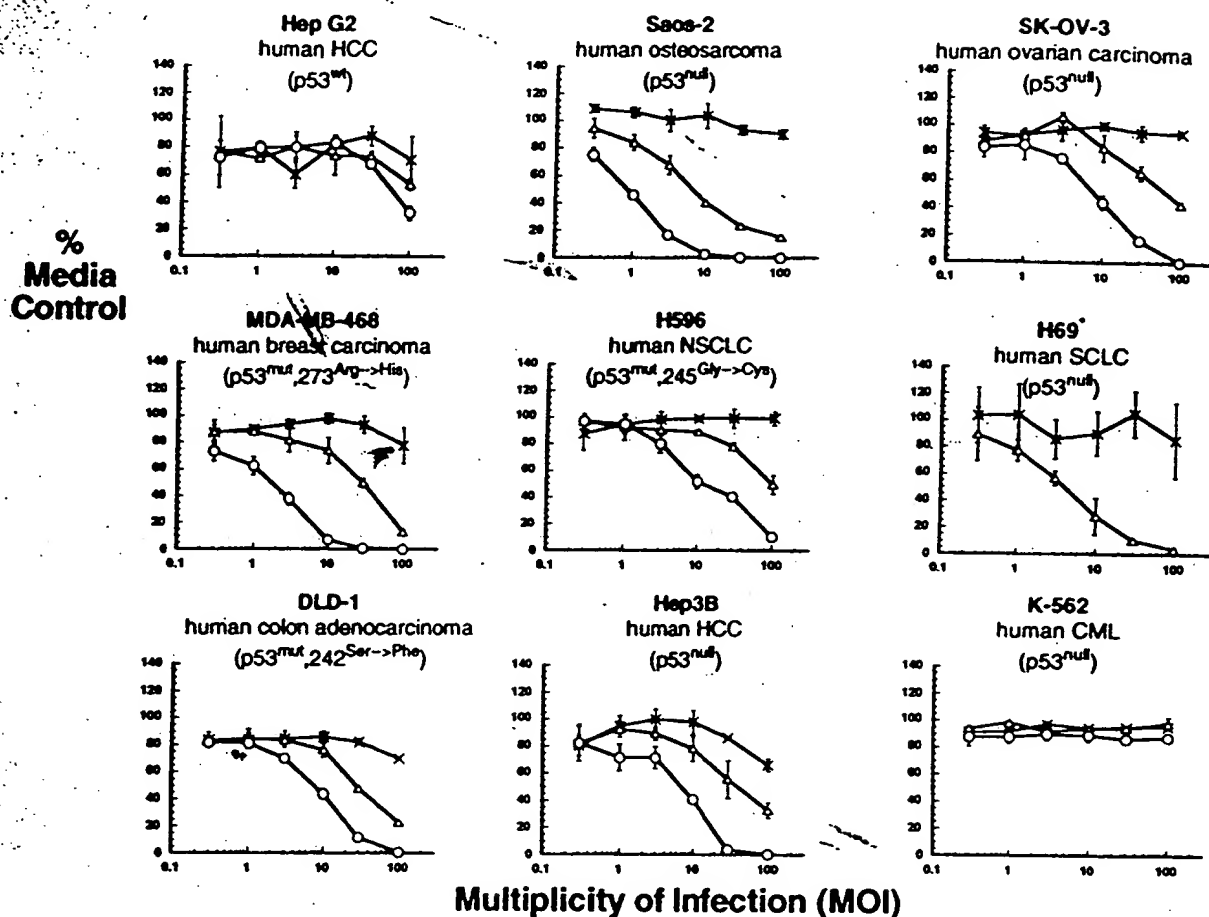


FIG. 4. p53-dependent inhibition of DNA synthesis in human tumor cell lines by A/M/N/53 and A/C/N/53. Nine different tumor cell lines were infected with either control adenovirus A/M/ (xx), or the p53-expressing A/M/N/53 ( $\Delta$ ) or A/C/N/53 (O) virus at increasing moi as indicated. Tumor type and p53 status are noted for each cell line (wt, wild type; null, no protein expressed; mut, mutant protein expressed). DNA synthesis was measured 72 hr post-infection as described in Materials and Methods. Results are from triplicate measurements at each dose (mean  $\pm$  SD), and are plotted as % of media control versus moi. (\*) H69 cells were only tested with A/M and A/M/N/53 virus.

pressed mutant p53 or failed to express p53 protein. We also found that in this assay, the A/C/N/53 construct was consistently more potent than the A/M/N/53. In Saos-2 (osteosarcoma) and MDA-MB468 (breast cancer) cells, nearly 100% inhibition of DNA synthesis was achieved with the A/C/N/53 construct at a moi as low as 10. At doses where inhibition by the control adenovirus is only 10–30%, we observed a 50–100% reduction in DNA synthesis using either p53 recombinant adenovirus. In contrast, we observed no significant p53-specific effect with either construct as compared to control virus in HEP G2 cells (hepatocarcinoma cell line expressing endogenous wild-type p53; Bressac *et al.*, 1990), nor in the K562 (p53 null; Feinstein *et al.*, 1992) leukemic cell line.

#### Tumorigenicity in nude mice

In a more stringent test of function for our p53 recombinant adenoviruses, we infected tumor cells *ex vivo* and then injected

the cells into nude mice to assess the ability of the recombinants to suppress tumor growth *in vivo*. Saos-2 cells infected with A/M/N/53 or control A/M virus at a moi of 3 or 30 were injected into opposite flanks of nude mice. Tumor sizes were then measured twice a week over an 8-week period. At a moi of 30, we did not observe any tumor growth in the p53-treated flanks in any of the animals, while the control treated tumors continued to grow (Fig. 5). The progressive enlargement of the control virus-treated tumors was similar to that observed in the buffer-treated control animals. We also observed a clear difference in tumor growth between the control adenovirus and the p53 recombinant at a moi of 3, although tumors from 2 out of the 4 p53-treated mice did start to show some growth after approximately 6 weeks (data not shown). Thus, the A/M/N/53 recombinant adenovirus is able to mediate p53-specific tumor suppression in an *in vivo* environment. We have also observed very similar results when infecting and injecting the NSCLC cell line H596, which expresses mutant p53 protein with the same viruses (unpublished observations).

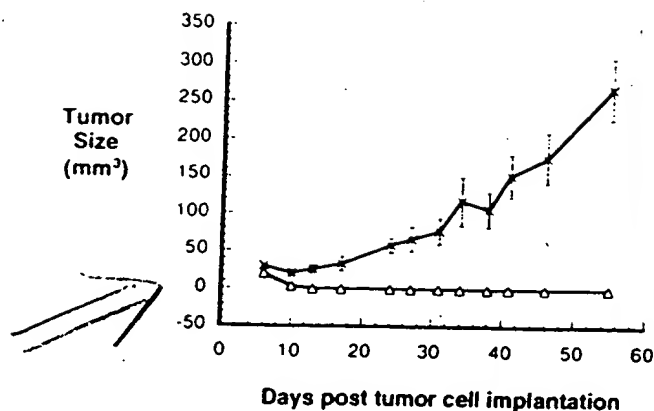


FIG. 5. Tumorigenicity of p53-infected Saos-2 cells in nude mice. Saos-2 cells were infected with either the control A/M virus or the p53 recombinant A/M/N/53 at moi = 30. Treated cells were injected subcutaneously into the flanks of nude mice, and tumor dimensions were measured (as described in Materials and Methods) twice per week for 8 weeks. Results are plotted as tumor size versus days post tumor cell implantation for both control A/M-(x) and A/M/N/53-(Δ) treated cells. Error bars represent the mean tumor size  $\pm$  SEM for each group of 4 animals at each time point.

#### In vivo expression of rAd/p53

Although *ex vivo* treatment of cancer cells and subsequent injection into animals provided a critical test of tumor suppression, a more clinically relevant experiment is to determine if injected p53 recombinant adenovirus could infect and express p53 in established tumors *in vivo*. To address this, H69 (SCLC, p53<sup>null</sup>) cells were injected subcutaneously into nude mice, and tumors were allowed to develop for 32 days. At this time, a single injection of  $2 \times 10^9$  pfu of either A/C/53 or A/C/ $\beta$ -Gal adenovirus was injected into the peritumoral space surrounding the tumor. Tumors were then excised at either day 2 or day 7 following the adenovirus injection, and poly(A) RNA was isolated from each tumor. RT-PCR, using recombinant-p53 specific primers, was then used to detect p53 mRNA in the p53-treated tumors (Fig. 6, lanes 1, 2, 4, 5). No p53 signal was evident from the tumors excised from the  $\beta$ -Gal-treated animals (Fig. 6, lanes 3 and 6). Amplification with actin primers served as a control for the RT-PCR reaction (Fig. 6, lanes 7–9), while a plasmid containing the recombinant-p53 sequence served as a positive control for the recombinant-p53-specific band (Fig. 6, lane 10). This experiment demonstrates that a p53 recombinant adenovirus can specifically direct expression of p53 mRNA within established tumors following a single injection into the peritumoral space. It also provides evidence for *in vivo* viral persistence for at least 1 week following infection with a p53 recombinant adenovirus.

#### In vivo efficacy

To address the feasibility of gene therapy of established tumors, a tumor-bearing nude mouse model was used. H69 cells were injected into the subcutaneous space on the right flank of mice, and tumors were allowed to grow for 2 weeks. Mice then received peritumoral injections of buffer or recombinant virus

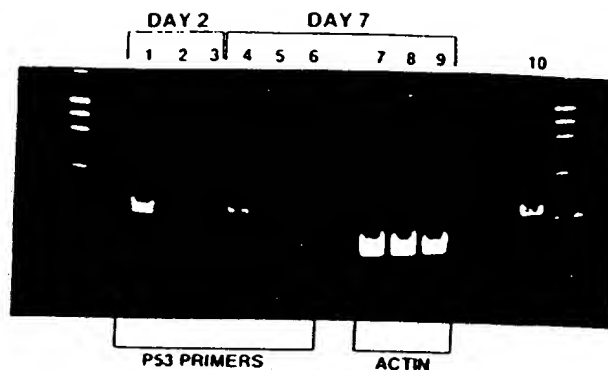


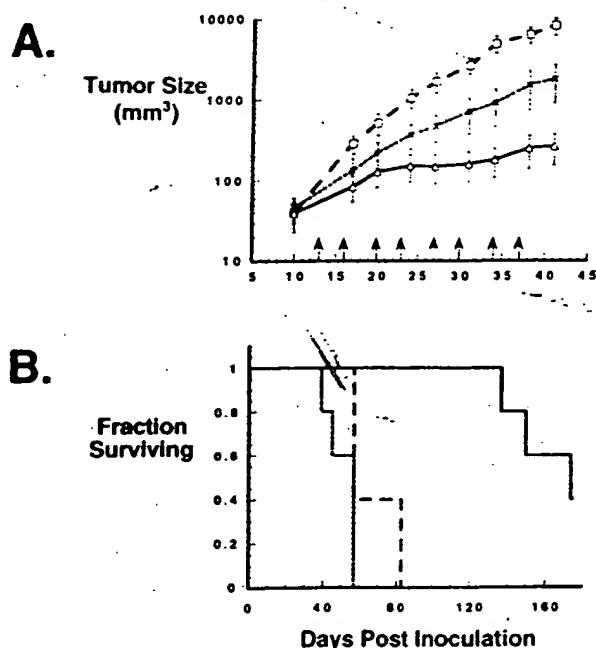
FIG. 6. Expression of rAd/p53 RNA in established tumors. H69 (SCLC) cells were injected subcutaneously into nude mice and allowed to develop tumors for 32 days until reaching a size of approximately 25–50 mm<sup>3</sup>. Mice were randomized and injected peritumorally with  $2 \times 10^9$  pfu of either control A/C/ $\beta$ -Gal or A/C/53 virus. Tumors were excised 2 and 7 days post injection, and poly(A) RNA was prepared from each tumor sample. RT-PCR was carried out using equal RNA concentrations and primers specific for recombinant p53 message. PCR amplification was for 30 cycles at 94°C 1 min, 55°C 1.5 min, 72°C 2 min, and a 10-min, 72°C final extension period in an Omnigen thermocycler (Hybaid). The PCR primers used were a 5' Tripartite Leader cDNA (5'-CGCCACCGAGGGACCT-GAGCGAGTC-3') and a 3' p53 primer (5'-TTCTGGGAAGG-GACAGAAGA-3'). Lanes 1, 2, 4, and 5, p53-treated samples excised at days 2 or 7 as indicated; lanes 3 and 6, from  $\beta$ -Gal-treated tumors; lanes 7, 8, and 9, replicates of lanes 4, 5, and 6, respectively, amplified with actin primers to verify equal loading; lane 10, a positive control using a tripartite/p53 containing plasmid.

twice weekly for a total of 8 doses. In the mice treated with buffer or control A/M virus, tumors continued to grow rapidly throughout the treatment, whereas those treated with the A/M/N/53 virus grew at a greatly reduced rate (Fig. 7A). Although control animals treated with buffer alone had accelerated tumor growth as compared to either virus-treated group, we found no significant differences in body weight among the three groups during the treatment period (data not shown). Tumor ulceration in some animals limited the relevance of tumor size measurements after day 42. However, continued monitoring of the animals to determine survival time demonstrated a survival advantages for the p53-treated animals (Fig. 7B). The last of the control adenovirus-treated animals died on day 83, while buffer alone treated controls had all expired by day 56. In contrast, all 5 animals treated with the A/M/N/53 survived up to day 137 before the first animal in this group died (Fig. 7B). Two animals continue to survive at day 174. Together, our data indicate a p53-specific effect on both tumor growth and survival time in animals with established p53-deficient tumors.

## DISCUSSION

#### Adenovirus vectors expressing p53

We have constructed recombinant human adenovirus vectors that are capable of expressing high levels of wild-type p53



**FIG. 7.** *In vivo* tumor suppression and increased survival time with A/M/N/53. H69 (SCLC) tumor cells were injected subcutaneously into nude mice and allowed to develop for 2 weeks. Peritumoral injections of either buffer alone (□), control A/M adenovirus (x), or A/M/N/53 (Δ) (both virus  $2 \times 10^9$  pfu/injection) were administered twice per week for a total of 8 doses. Tumor dimensions were measured twice per week and tumor volume was estimated as described in Materials and Methods. A. Tumor size is plotted for each virus *versus* time (days) post inoculation of H69 cells. Error bars indicate the mean tumor size  $\pm$  SEM for each group of 5 animals. Arrows indicate days of virus injections. B. Mice were monitored for survival and the fraction of mice surviving per group *versus* time post inoculation of buffer alone (—), control A/M (—), or A/M/N/53 (—) virus-treated H69 cells is plotted.

protein in a dose-dependent manner. Each vector contains deletions in the E1a and E1b regions that render the virus replication deficient (Challberg and Kelly, 1979; Horowitz, 1991). Of further significance is that these deletions include those sequences encoding the E1b 19- and 55-kD proteins. The 19-kD protein is reported to be involved in inhibiting apoptosis (Rao *et al.*, 1992; White *et al.*, 1992), whereas the 55-kD protein is able to bind wild-type p53 protein (Samow *et al.*, 1982; Heuvel *et al.*, 1990). By deleting these adenoviral sequences, we remove potential inhibitors of p53 function through direct binding to p53 or potential inhibition of p53-mediated apoptosis. We have created additional constructs that have had the remaining 3' E1b sequence, including all protein IX coding sequence, deleted as well. Although this has been reported to reduce the packaging size capacity of adenovirus to approximately 3 kb, less than wild-type virus (Ghosh-Choudhury *et al.*, 1987), these constructs are also deleted in the E3 region so that the A/M/N/53 and A/C/N/53 constructs are well within this size range. By deleting the pIX region, adenoviral sequences homologous to those contained in 293 cells are reduced to approximately 300 bp, decreasing the chances of regenerating replication-competent

wild-type adenovirus through recombination. Constructs lacking pIX coding sequence appear to have equal efficacy and drive equivalent levels of p53 protein expression as those with pIX (unpublished observations).

#### p53/Adenovirus efficacy *in vitro*

In concordance with a strong dose dependency for expression of p53 protein in infected cells, we have also demonstrated a dose-dependent, p53-specific inhibition of tumor cell growth by our recombinants. We were able to inhibit cell division, demonstrated by the inhibition of DNA synthesis, in a wide variety of tumor cell types known to lack wild-type p53 protein expression. Bacchetti and Graham (1993) recently reported p53-specific inhibition of DNA synthesis in the ovarian carcinoma cell line SKOV-3 by a p53 recombinant adenovirus in similar experiments. In addition to ovarian carcinoma, we have demonstrated that additional human tumor cell lines, representative of clinically important human cancers and including lines overexpressing mutant p53 protein, can also be growth inhibited by our p53 recombinants. At moi values where the A/C/N/53 recombinant is 90–100% effective in inhibiting DNA synthesis in these tumor types, control adenovirus-mediated suppression is less than 20%.

Although Feinstein *et al.* (1992) reported that reintroduction of wild-type p53 could induce differentiation and increase the proportion of cells in G<sub>1</sub> *versus* S + G<sub>2</sub> for leukemic K562 cells, we found no p53-specific effect in this line. Horvath and Weber (1988) have reported that human peripheral blood lymphocytes are highly nonpermissive to adenovirus infection. In separate experiments, we found that we were not able to infect the nonresponding K562 cells significantly with recombinant A/C/β-Gal adenovirus, while other cell lines, including the control Hep G2 line and those showing a strong p53 effect, were readily infectable (Harris *et al.*, in preparation). Thus, at least part of the variability of efficacy would appear to be due to variability of infection, although other factors may be involved as well. For example, Chen *et al.* (1991) reported that wild-type p53 can suppress tumorigenicity without inhibiting the growth rate of some tumor lines. Alternatively, mutations of regulatory proteins acting downstream from p53 may also exist in some tumor cell lines, limiting the effect of p53 treatment. The lack of a p53-specific effect in the wild-type control cell line Hep G2 is encouraging, suggesting that overexpression of wild-type p53 over endogenous background levels may have only minor effects in normal cells infected with the recombinant.

The ability to treat human cancer cells *ex vivo* and suppress their growth *in vivo* when implanted into an animal is an important step toward identifying promising gene therapy candidates. The results observed with the A/M/N/53 virus in Fig. 5 demonstrates that complete suppression is possible in an *in vivo* environment. The resumption of tumor growth in 2 out of the 4 p53-treated animals at the lower moi most likely resulted from a small percentage of cells not initially infected with the p53 recombinant at this dose. We did not analyze the resulting tumors for the presence of adenoviral genomes. The complete suppression seen with A/M/N/53 at the high dose, however, shows that the ability of tumor growth to recover can be overcome.

### p53/Adenovirus *in vivo* efficacy

Work presented here and by other groups (Chen *et al.*, 1990; Takahashi *et al.*, 1992) have shown that human tumor cells lacking expression of wild-type p53 can be treated *ex vivo* with p53 and result in suppression of tumor growth when the treated cells are transferred into an animal model. This report presents the first evidence of tumor suppressor gene therapy of an *in vivo* established tumor, resulting in both suppression of tumor growth and increased survival time. Delivery to tumor cells did not rely on direct injection into the tumor mass. Rather, p53 recombinant adenovirus was injected into the peritumoral space, and p53 mRNA expression was detected within the tumor. p53 expressed by the recombinants was functional and strongly suppressed tumor growth as compared to that of control, non-p53-expressing adenovirus-treated tumors. However, both p53 and control virus-treated tumor groups showed tumor suppression as compared to buffer-treated controls. It has been demonstrated that local expression of tumor necrosis factor (TNF), interferon- $\gamma$  (IFN- $\gamma$ ), interleukin (IL)-2, IL-4, or IL-7 can lead to T-cell-independent transient tumor suppression in nude mice (Hoch *et al.*, 1992). Exposure of monocytes to adenovirus results in the release of TNF, and adenovirus virions are also weak inducers of IFN- $\alpha/\beta$  (for review, see Gooding and Wold, 1990). Therefore, it is not surprising that we observed some tumor suppression in nude mice even with the control adenovirus. We did not observe this virus-mediated tumor suppression in the *ex vivo* control virus-treated Saos-2 tumor cells described earlier. The p53-specific *in vivo* tumor suppression was dramatically demonstrated by continued monitoring of the animals in Fig. 7. The survival time of the p53-treated mice was significantly increased, with 5 out of 5 animals still alive more than 135 days after tumor cell inoculation compared to 0 out of 5 adenovirus control-treated animals. Two out of 5 mice continue to survive beyond day 170, more than twice the survival time of the longest-lived control virus and buffer-treated animals. The surviving animals still exhibit growing tumors, which may reflect cells not initially infected with the p53 recombinant adenovirus. Higher or more frequent dosing schedules may address this. In addition, promoter shutoff (Palmer *et al.*, 1991) or additional mutations may have rendered these cells resistant to the p53 recombinant adenovirus treatment.

### Implications for gene therapy

There will be over one million new cases of cancer diagnosed this year, and half that number of cancer-related deaths (American Cancer Society, 1993). p53 mutations are the most common genetic alteration associated with human cancers, occurring in 50–60% of human cancers (Bartek *et al.*, 1991; Hollstein *et al.*, 1991; Levine, 1993). The goal of gene therapy in treating p53-deficient tumors is to reinstate a normal, functional copy of the wild-type p53 gene so that control of cellular proliferation is restored. p53 plays a central role in cell cycle progression, arresting growth so that repair or apoptosis can occur in response to DNA damage. The possibility of using p53/adenovirus to drive tumor cells into the apoptotic pathway is intriguing. Wild-type p53 has recently been identified as a necessary component for apoptosis induced by irradiation or

treatment with some chemotherapeutic agents (Lowe *et al.*, 1993a,b). Due to the high prevalence of p53 mutations in human tumors, it is possible that tumors which have become refractory to chemotherapy and irradiation treatments may have become so due in part to the lack of wild-type p53. By resupplying functional p53 to these tumors, it is possible that they will now become susceptible to apoptosis normally associated with the DNA damage induced by radiation and chemotherapy.

One of the critical points in successful human tumor suppressor gene therapy is the ability to affect a significant fraction of the cancer cells. Toward that goal, recombinant adenoviruses have distinct advantages over other gene delivery methods (for review, see Siegfried, 1993). Adenoviruses have never been shown to induce tumors in humans and have been safely used as live vaccines (Straus, 1984). Replication-deficient recombinant adenoviruses can be produced by replacing the E1 region necessary for replication with the target gene. Adenovirus does not integrate into the human genome as a normal consequence of infection, thereby greatly reducing the risk of insertional mutagenesis possible with retrovirus or AAV vectors. This lack of stable integration also leads to an additional safety feature in that the transferred gene effect will be transient, as the extrachromosomal DNA will be gradually lost with continued division of normal cells. Stable, high-titer recombinant adenovirus can be produced at levels not achievable with retrovirus or AAV, allowing enough material to be produced to treat a large patient population. Others have shown that adenovirus-mediated gene delivery has a strong potential for gene therapy for diseases such as cystic fibrosis (Rosenfeld *et al.*, 1992; Rich *et al.*, 1993) and  $\alpha_1$ -antitrypsin deficiency (Lemarchand *et al.*, 1992). Although other alternatives for gene delivery, such as cationic liposome-DNA complexes, are also currently being explored, none as yet appear as effective as adenovirus-mediated gene delivery.

Here, we have shown that recombinant adenoviruses expressing wild-type p53 can efficiently inhibit DNA synthesis and suppress the growth of a broad range of human tumor cell types, including clinically relevant targets. Furthermore, we have shown that the recombinant adenoviruses can express p53 in an *in vivo* established tumor without relying on direct injection into the tumor or prior *ex vivo* treatment of the cancer cells. The p53 expressed is functional and effectively suppressed tumor growth *in vivo* and significantly increased survival time in a nude mouse model of human lung cancer. Although further studies are needed to ensure the safety of this method of gene delivery and address possible problems of immune responses, the data presented here strongly support the concept of adenovirus-mediated p53 gene therapy of p53-deficient tumors in humans.

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## REFERENCES

- AMERICAN CANCER SOCIETY. (1993). Cancer Facts and Figures. BACCHETTI, S., and GRAHAM, F. (1993). Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. *Int. J. Oncol.* 3, 781-788.
- BAKER S.J., MARKOWITZ, S., FEARON, E.R., WILLSON, J.K.V., and VOGELSTEIN, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 249, 912-915.
- BARTEK, J., BARTKOVA, J., VOJTESEK, B., STASKOVA, Z., LUKAS, J., REJTHAR, A., KOVARIK, J., MIDGLEY, C.A., GANNON, J.V., and LANE, D.P. (1991). Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene* 6, 1699-1703.
- BRESSAC, B., GALVIN, K.M., LIANG, T.J., ISSELBACHER, K.J., WANDS, J.R., and OZTURK, M. (1990). Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* 87, 1973-1977.
- CHALLBERG, M.D., and KELLY, T.J. (1979). Adenovirus DNA replication in vitro. *Biochemistry* 18, 655-659.
- CHEN P.L., CHEN Y., BOOKSTEIN R., and LEE W.H. (1990). Genetic mechanisms of tumor suppression by the human p53 gene. *Science* 250, 1576-1580.
- CHEN, Y., CHEN, P.L., ARNAIZ, N., GOODRICH, D., and LEE, W.H. (1991). Expression of wild-type p53 in human A673 cells suppresses tumorigenicity but not growth rate. *Oncogene* 6, 1799-1805.
- CHENG, J., YEE, J.K., YEARGIN, J., FRIEDMANN, T., and HAAS, M. (1992). Suppression of acute lymphoblastic leukemia by the human wild-type p53 gene. *Cancer Res.* 52, 222-226.
- DILLER, L., KASSEL, J., NELSON, C.E., GRYKA, M.A., LITWAK, G., GEBHARDT, M., BRESSAC, B., OZTURK, M., BAKER, S.J., VOGELSTEIN, B., and FRIEND, S.H. (1990). p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.* 10, 5772-5781.
- FEINSTEIN, E., GALE, R.P., REED, J., and CANAANI, E. (1992). Expression of the normal p53 gene induces differentiation of K562 cells. *Oncogene* 7, 1853-1857.
- GHOSH-CHOUDHURY, G., HAJ-AHMAD, Y., and GRAHAM, F.L. (1987). Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *EMBO J.* 6, 1733-1739.
- GOODING, L.R., and WOLD, W.S.M. (1990). Molecular mechanisms by which adenoviruses counteract antiviral immune defenses. *Crit. Rev. Immunol.* 10, 53-71.
- GRAHAM, F.L., and VAN DER ERB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456-467.
- GRAHAM, F.L., SMILEY, J., RUSSELL, W.C., and NAIRN, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, 59-74.
- GRAHAM, F.L., and PREVEC, L. (1991). Manipulation of adenovirus vectors. In *Methods in Molecular Biology*, vol. 7. *Gene Transfer and Expression Protocols*. E.J. Murray, ed. (The Humana Press Inc., Clifton NJ) pp. 109-128.
- HEUVEL, S.J.L., LAAR, T., KAST, W.M., MELIEF, C.J.M., ZANTEMA, A., and VAN DER EB, A.J. (1990). Association between the cellular p53 and the adenovirus 5 E1B-55kd proteins reduces the oncogenicity of Ad-transformed cells. *EMBO J.* 9, 2621-2629.
- HOCK, H., DORSCH, M., KUZENDORF, U., QIN, Z., DIAMANTSTEIN, T., and BLANKENSTEIN, T. (1992). Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon  $\gamma$ . *Proc. Natl. Acad. Sci. USA* 90, 2774-2778.
- HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B., and HARRIS C. (1991). p53 mutations in human cancers. *Science* 253, 49-53.
- HOROWITZ, M.S. (1991). Adenoviridae and their replication. In *Fields Virology*. B.N. Fields, ed. (Raven Press, New York) pp. 1679-1721.
- HORVATH, J., and WEBER, J.M. (1988). Nonpermissivity of human peripheral blood lymphocytes to adenovirus type 2 infection. *J. Virol.* 62, 341-345.
- HSU, I.C., TOKIWA, T., BENNETT, W., METCALF, R.A., WELSH, J.A., SUN, T., and HARRIS, C.C. (1993). p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. *Carcinogenesis* 14, 987-992.
- JONES, N., and SHENK, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 17, 683-689.
- KEURBITZ, S.J., PLUNKETT, B.S., WALSH, W.V., and KASTAN, M.B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA* 89, 7491-7495.
- LANE, D.P. (1992). p53, guardian of the genome. *Nature* 358, 15-16.
- LEMARCHAND, P., JAFFE, H.A., DANIEL, C., CID, M.C., KLEINMAN, H.K., STRATFORD-PERRICAUDET, L.D., PERRICAUDET, M., PAVIRANI, A., LECOCQ, J.P., and CRYSTAL, R.G. (1992). Adenovirus-mediated transfer of a recombinant human  $\alpha_1$ -antitrypsin cDNA to human endothelial cells. *Proc. Natl. Acad. Sci. USA* 89, 6482-6486.
- LEVINE, A.J. (1993). The tumor suppressor genes. *Annu. Rev. Biochem.* 62, 623-651.
- LOWE, S.W., RULEY, H.E., JACKS, T., and HOUSMAN, D.E. (1993a). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 74, 957-967.
- LOWE, S.W., SCHMITT, E.M., SMITH, S.W., OSBORNE, B.A., and JACKS, T. (1993b). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362, 847-852.
- MERCER, W.E., SHIELDS, M.T., AMIN, M., SUAVE, G.J., APPELLA, E., ROMANO, J.W., and ULLRICH, S.J. (1990). Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA* 87, 6166-6170.
- PALMER, T.D., ROSMAN, G.J., OSBORNE, W.R., and MILLER, A.D. (1991). Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc. Natl. Acad. Sci. USA* 88, 1330-1334.
- RAO, L., DEBBAS, M., SABBATINI, P., HOCKENBERY, D., KORSMEYER, S., and WHITE, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA* 89, 7742-7746.
- RICH, D.P., COUTURE, L.A., CARDOZA, L.M., GUIGGIO, V.M., ARMENTANO, D., ESPINO, P.C., HEHIR, K., WELSH, M.J., SMITH, A.E., and GREGORY, R.J. (1993). Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis. *Hum. Gene Ther.* 4, 461-476.
- ROSENFELD, M.A., YOSHIMURA, K., TRAPNELL, B.C., YONEYAMA, K., ROSENTHAL, E.R., DALEMANS, W., FUKAYAMA, M., BARGON, J., STIER, L.E., STRATFORD-PERRICAUDET, L., PERRICAUDET, M., GUGGINO, W.B., PAVIRANI, A., LECOCQ, J.P., and CRYSTAL, R.G. (1992). In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* 68, 143-155.
- SAMBROOK, J., FRITSCH, E.F., and MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).
- SARNOW, P., HO, Y.S., WILLIAMS, J., and LEVINE, A.J. (1982).

- Adenovirus E1b-58 kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell* 28, 387-394.
- SHAW, P., BOVEY, R., TARDY, S., SAHLI, R., SORDAT, B., and COSTA, J. (1992). Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. USA* 89, 4495-4499.
- SIEGFRIED, W. (1993). Perspectives in gene therapy with recombinant adenoviruses. *Exp. Clin. Endocrinol.* 101, 7-11.
- STRAUS, S.E. (1984). Adenovirus infections in humans. In *The Adenoviruses*. H.S. Ginsberg, ed. (Plenum Press, New York) pp. 451-496.
- TAKAHASHI, T., NAU, M.M., CHIBA, I., BIRRER, M.J., ROSENBERG, R.K., VINOCOUR, M., LEVITT, M., PASS, H., GAZDAR, A.F., and MINNA, J.D. (1989). p53: A frequent target for genetic abnormalities in lung cancer. *Science* 246, 491-494.
- TAKAHASHI, T., CARBONE, D., TAKAHASHI, T., NAU, M.M., HIDA, T., LINNOILA, I., UEDA, R., and MINNA, J.D. (1992). Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. *Cancer Res.* 52, 2340-2343.
- THIMMAPAYA, B., WEINBERGER, C., SCHNEIDER, R.J., and SHENK, T. (1982). Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. *Cell* 31, 543-551.
- WANG, A.M., DOYLE, M.V., and MARK, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 86, 9717-9721.
- WHITE, E., SABBATINI, P., DEBBAS, M., WOLD, W.S.M., KUSHER, D.I., and GOODING, L.R. (1992). The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor. *Mol. Cell. Biol.* 12, 2570-2580.
- YONISH-ROUACH, E., RESNITZKY, D., LOTEM, J., SACHS, L., KIMCHI, A., and OREN, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 352, 345-347.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

*In re* Application of:  
GARY L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHOD AND COMPOSITION FOR  
THE DIAGNOSIS AND TREATMENT OF  
CANCER

Group Art Unit: 1632

Examiner: K. Hauda

Atty. Dkt. No.: INRP:041/HYL

CERTIFICATE OF MAILING  
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:

8/12/98  
DATE

SIGNATURE

DECLARATION OF DR. GARY L. CLAYMAN UNDER 37 C.F.R. § 1.131

Hon. Assistant Commissioner for Patents  
Washington, D.C. 20231

I, Gary L. Clayman, D.D.S., M.D., declare that:

1. I am a U.S. citizen residing at 6353 Westchester Street, Houston, Texas. I am Associate Professor of Surgery and Deputy Chairman of the Department of Head and Neck Surgery at the University of Texas M.D. Anderson Cancer Center. A copy of my curriculum vitae outlining my education and research training is attached (Exhibit A).

2. I am the inventor of the above-captioned application and a portion of my research has been sponsored by Introgen Therapeutics, Inc., a company that has licensed this technology.

3. I am a co-author of Clayman *et al.*, published in *Cancer Research* (Exhibit B), along with Drs. Adel K. El-Naggar, Jack A. Roth, Wei-Wei Zhang, Helmuth Goepfert, Dorothy L. Taylor, and Ta-Jen Liu. I also am co-author of Liu *et al.*, published in *Cancer Research* (Exhibit C), along with Drs. Liu, El-Naggar, Taylor, Timothy J. McDonnell, Kim D. Steck, and Mary Wang.

4. Drs. El-Naggar, Roth, Zhang, Goepfert, Taylor, Liu, McDonnell, Steck and Wang, the non-inventor co-authors of this paper, did not contribute to the conception of the present invention of using Adp53 for the treatment of head and neck cancer. Each of these individuals acted under the supervision and direction of myself in generating the results reported in these papers, or as a reviewer of the manuscripts prior to publication.

5. Dr. Adel K. El-Naggar performed the pathologic analysis in Exhibit B and the fluorescent analysis in Exhibit C.

6. Dr. Jack A. Roth provided the Adp53 vector in Exhibits B and C.

7. Dr. Wei-Wei Zhang developed the Adp53 vector in Exhibit B.

8. Dr. Helmuth Goepfert reviewed the Exhibit B manuscript prior to publication.

9. Dr. Dorothy L. Taylor developed and maintained the head and neck cancer cell lines, and assisted in the experiments described in Exhibits B and C.

10. Dr. Ta-Jen Liu performed the *in vitro* propagation of the Adp53 vector in Exhibit B and performed the DNA fragmentation analysis in Exhibit C.

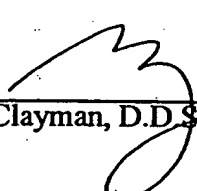
11. Dr. Timothy J. McDonnell reviewed the Exhibit C manuscript prior to publication.

12. Dr. Kim D. Steck performed the technical fluorescent sorting studies in Exhibit C.

13. Dr. Mary Wang performed infection assays and viral propagation in Exhibit C.

14. I hereby declare that all statements made herein of my knowledge are true and that all statements made herein on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the U.S. Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/18/98  
Date

  
Gary L. Clayman, D.D.S., M.D.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:  
GARY L. CLAYMAN

Serial No.: 08/758,033

Filed: November 27, 1996

For: METHOD AND COMPOSITION FOR  
THE DIAGNOSIS AND TREATMENT OF  
CANCER

Group Art Unit: 1632

Examiner: K. Hauda

Atty. Dkt. No.: INRP:041/HYL

INVENTOR'S DECLARATION UNDER 37 C.F.R. § 1.131

Hon. Commissioner of Patents  
Washington DC 20231

I, Gary L. Clayman, declare as follows:

1. I am the sole inventor of the subject matter of all claims currently pending in the referenced patent application.
2. It is my understanding that the Patent and Trademark Office Examiner in charge of the above-captioned application has advanced a rejection of the claims over the references Katayose *et al.*, "Cytotoxic effects of adenovirus-mediated wild-type p53 protein expression in normal and tumor mammary epithelial cells" *Clinical Cancer Research*. 1:889-897 (1995) and Srivastava *et al.*, "Recombinant adenovirus vector expressing wild-type p53 is a potent inhibitor of prostate cancer cell proliferation" *Urology*. 46:843-848 (1995).

3. These articles are in no way relevant to the patentability of my invention, which is directed to the treatment of human cancer patients with the p53 gene. First, both papers cite data from *in vitro* experiments using various cancer cell lines. They do not teach the therapeutic benefits towards humans with the addition of Ad-WTp53. Since the data presented in this invention clearly shows the therapeutic benefits of Ad-WTp53 towards humans, these papers are irrelevant. Secondly, the data is inconsistent further suggesting the unpredictability of human treatment. For example, Srivastava *et al.* showed the addition of Ad-WTp53 negatively affected cell growth *in vitro* but positively affected growth *in vivo*. One is unable to deduce from this contradictory data how Ad-WTp53 would affect human treatment. Furthermore, both papers teach that there are differences between tumor cells that contain mutated p53 and tumor cells that contain wild-type p53. Katayose *et al.* demonstrated that Ad-WTp53 treatment on cells that are deficient of p53 or that contain a mutated p53 gene inhibit cell growth and induce apoptosis. However, they suggest that wild-type p53 tumor cell growth or apoptosis is not affected by the addition of Ad-WTp53. This teaches away from my invention, which is to determine the effects of Ad-WTp53 on wild-type p53 tumor cells.

4. Furthermore, I had fully developed the concept of my invention well before the publication of the forgoing articles. I understand that the earliest of these two articles, Katayose *et al.*, was mailed by the publisher on August 11, 1995. Prior to this date I had fully conceived of using the p53 gene in clinical therapy of human cancer patients having tumors expressing wild-type p53 ("wild-type p53 tumors"). Furthermore, I was diligent in seeking to reduce this invention to practice from August 11, 1995 at least up until the filing of the priority application on November 30, 1995 (US 60/007810).

5. My conception of this invention and diligence is supported by following evidence:

A. Attached as Exhibit 1 is the transcribed tape of my Grand Rounds Seminar dated prior to August 11, 1995 in which I presented my intentions to treat human cancer patients having either wild-type p53 tumors or tumors that contain a mutated p53 gene with an adenovirus carrying wild-type p53 ("Ad-WTp53") (see highlighted text).

B. At that same time, I was working towards obtaining the necessary approvals from the Food and Drug Administration ("FDA"), the National Institutes of Health Recombinant Advisory Committee ("NIH/RAC") and from the institutional review board of MD Anderson Cancer Center ("IRB"), to conduct a clinical trial of the Ad-WTp53 in human cancer patients having both wild-type and mutant p53 tumors.

C. Attached as Exhibit 2 are various approvals received from MD Anderson Cancer Center administration, the IRB and the institutional biosafety committee. While dates have been redacted from these documents, each is dated prior to August 11, 1995.

D. Attached as Exhibit 3 is the final version of the approved informed consent form. This version is redacted to remove names and dates, but this version of the form is dated prior to August 11, 1995.

E. A proposed protocol for the clinical study, designated HNS 94-001, was submitted to the FDA and NIH/RAC prior to August 11, 1995. However, various revisions were required to be made to the protocol during the approval process, and such revisions are entered to the protocol, and dated, on a page-by-page basis. I have attached

the final, approved protocol for HNS 94-001 as Exhibit 4. I should note that all of the dates prior to August 11, 1995 have been redacted from this document. However, an asterisk ("\*") has been placed on the upper right corner of each page that is dated prior to August 11, 1995. Pages that were revised after August 11, 1995 are not date-redacted.

F. In this protocol I observe that our laboratory studies had shown that head and neck squamous cell carcinomas ("HNSCC") underwent apoptosis (cell death) when treated with Ad-WTp53, regardless of endogenous p53 status. See Protocol, page 3, second full paragraph. For this reason, the study was designed to include patients having HNSCC regardless of p53 endogenous status of the tumor, and to assess the tumor for its p53 status. See, *e.g.*, Protocol, page 11, section 6.7.

G. On August 24, 1995 I received initial approval from the FDA for the Investigational New Drug ("IND") study with some revisions. See Exhibit 5. The requested revisions were entered into the protocol.

H. On September 25, 1995, I sought administrative approval for the revised protocol. Attached as Exhibit 6 is the approval request with attached revision pages.

I. On October 4, 1995 I received administrative approval for the revised protocol. See Exhibit 7.

J. On October 10, 1995, the revised "Clinical protocol for modification of tumor suppressor gene expression and induction of apoptosis in head and neck squamous cell carcinoma (HNSCC) with an adenovirus vector expressing wildtype p53" (HNS 94-

001) was approved for activation and patient accrual by the Associate Vice President for Clinical and Translational Research (Dr. Leonard A. Zellwing). See Exhibit 8.

K. On October 16, 1995, the surveillance committee report noted that the study had received IRB approval, informed consent approval and FDA approval, but that it was still awaiting NIH/RAC approval. See Exhibit 9.

L. On October 25, 1995, we treated our first patient under the protocol. Treatment of this patient, and enrollment of other patients in the study, continued through the month November of 1995 and beyond, with study close-out occurring in the summer of 1997. See Exhibit 10.

6. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

May 21, 2002  
Date

Gary L. Clayman  
Gary L. Clayman



Clinical Grand Rounds at M.D. Anderson  
The University of Texas MD Anderson Cancer Center  
"Developing Novel Molecular Therapy for Head and Neck Cancer"\*

Dr. Gary L. Clayman, MD  
Director, Basic Science Research  
Department of Head and Neck Surgery

It's a real honor to have the opportunity to present oncology grand rounds here at M.D. Anderson and I appreciate the introduction by Dr. Gefford and if I can have the first slide please. The title of this talk is developing novel molecular therapy for head and neck cancer. This doesn't necessarily just apply to head and neck cancer. We're using head and neck cancer as a model, but certainly my particular clinical interest has driven me towards this particular entity. There are several clinical problems, or clinical dilemmas that occur in the management of patients that have head and neck cancer. The particular head and neck cancer that we are focusing our research efforts on is squamous cell cancer of the upper digestive tract.

To just refresh those that are minimally or nonfamiliar, local regional control remains a major problem in patients afflicted with this cancer. With only 10-15% of patients dying of this disease alone. The local regional failure continues to remain a major problem. Second primary cancers occur in this patient population probably a rate approximating 4-7% a year. This is really more a problem in patients that have early stage disease. Those patients with advance stage disease tend to succumb to their own disease process. Dissecting the molecular cascade that's occurring in the progression of head and neck cancer is critical in understanding this process, and it's probably the most humbling thing with regard to this particular type of cancer is that we truly have not impacted upon the overall and disease specific survival in patients that are afflicted with this disease.

I'd just like to show you this slide. It was a study that we did here a couple of years ago that looked at patients that were undergoing organ preservation attempts in studies that were directed under the offices of Dr. Wang-Kay Hong, and in this study, patients with advanced hyperthyroidal or laryngeal cancers that would require laryngectomy received chemotherapy and radiation therapy in attempts to preserve their organs. We therefore sought to determine whether these contemporary management how compared to our respective controls that had been treated in years gone past. As you can see in the disease specific survival, we do know better. These patients that had organ preservation attempts also had surgical salvage if they had failure. But basically stating this is just sort of a depiction that we do know better than we did 20-30 years ago.

There may be many different strategies for novel gene therapy in the management of solid organ tumors and I just gave a list of several of them here. One of them may be the delivery of a toxic gene or potentially a metabolite. The HSDTK gene if you've been reading the literature, is one that may represent this particular category. In that particular means of therapy, you would deliver a particular gene and then deliver either it itself would be toxic or something,

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\* Transcribed from a VCR tape of the Clinical Grand Rounds presentation of Dr. Clayman at the University of Texas MD Anderson Cancer Center. The VCR tape includes the figures that are referred to in this talk and can be made available upon request.

for example, with gancyclovir therapy HSDTK gene expression induces cell death. You may be able to augment immunogenicity of a tumor by gene therapy, by enhancing some particular cell membrane or characteristic of a tumor that can be monitored by the immune system or conversely we may be able to regulate the immune system either ex vivo or in vivo. You may be able to alter the chemosensitivity or radiation sensitivity of a tumor. In a recent publication by Dr. Wei-Wei Zhang and Roth et al. from this institution they showed that chemosensitivity may be augmented by a particular gene therapy. You may be able to reverse early molecular events in the carcinogenic cascade, and in this we're presently pursuing efforts that we're not going to talk about today, but you may be able to reverse the premalignant cascade in order to develop a prevention strategy in the management of this process. And, lastly, if you can target the particular cell cycle regulators or induce apoptosis in tumor cells, you may be able to regulate tumor cells by intervening in these steps.

We're particularly attracted to a particular kind of gene therapy or molecular vector that's an adenovirus vector. One of the attractions of this is its natural tropism for zero digestive tract epithelium and here we hope that we could adequately transduce the cells of interest. In other organ tissue systems, high transduction efficiency has been shown with these particular vectors and importantly if you delete particular components of the adenovirus and in particular E1A and E1B components you can render it replication defective and in this manner you can obviate some of the problems with permanent integration of extraneous components into the genome.

In our initial experiments we thought to determine whether we could simply transduce cells. And here we used a particular marker and this marker is the  $\beta$ -galactosidase marker. It's a  $\beta$ -galactosidase on an adenovirus replication defective vector. And in a dose response experiment, we sought to determine the transduction efficacy in squamous cell cancer cell lines. Here we have a mock infected cell line where we see no  $\beta$ -galactosidase expression and here we see at a 50 multiplicity of infection we see simply 50 viral particles per tumor cell, we see approximately 40-50% transduction efficiency. With higher viral titers here 100 multiplicity of infection, we see essentially our maximal infection rate of about 60-65%. With repeat infection, we see 100% transduction of these tumor cells. When we graphically depict this, independent of the tumor cell line and we plateau here this is with a single infection 24 hours after infection, our  $\beta$ -galactosidase expression is approximately 65-70%. Dr. Wei-Wei Zhang and Dr. Roth of this institution have collaborated with us in these studies. Drs. Wei-Wei Zhang and Roth have developed a wild-type p53 virus vector. Here in a linear diagram for you is the construction schematic for CMV promoter sites, the wild-type p53 cDNA and a polyadenylation site and the cell is replication defective at A1A in a component of B1B deletion.

Here are initial experiments that we sought to determine with this particular adenovirus vector that Dr. Roth generously gave to us so that we could induce mRNA expression in squamous cell head and neck cell lines. In these experiments the 293 packaging cell line that the virus is propagated in we see the positive control. In our negative control we see no expression of the p53 mRNA. In these experiments we used the parental cell line and we use a replication defective virus without any particular gene or promoter attached and then the wild-type p53 adenovirus vector. And here we see a small induction of endogenous but a very significant exogenous mRNA expression replication defective virus and the parental cell line controls with no increased expression. And again this was independent of the cell line that's utilized parental

control replication defective virus and here the infected cell line. Message is induced and we hope that protein would be induced also here in Western blot experiments with the same type of experimental schematic for packaging cell lines with significant expression of the p53 protein here the parental cell lines in very low levels of p53 protein. Both of the cell lines by the way are mutated for p53. This cell line treated with replication defective virus and here the adenovirus infected with the p53 and a very significant induction of p53 protein. Again, independent of the cell line that was utilized. From a light microscopic standpoint we sought to using immunohistochemistry to verify this protein expression as well as our transduction efficacy as we saw with the  $\beta$ -galactosidase virus. Here, the mock infected control, this is again, this is a mutated p53 cell line, here infected with the adenovirus p53 we see the characteristic nuclear staining that we would expect to see with the exogenous p53 expression. Again, the transduction efficiency approximates that that we saw with the  $\beta$ -galactosidase adenovirus expression vector.

Dr. Wei-Wei Zhang performed these experiments in order to determine the duration of this transient expression of p53 in infected cells. Here in a time course experiment over 15 days essentially no p53 is expressed two weeks following this transient expression with the p53 adenovirus vector.

In in vitro growth curve experiments we then sought to determine the effects on in vitro cell growth. Here is a mutated p53 cell line, we use mock infection in order to determine a routine growth rate in a logarithmic growth rate is seen. Here, a replication defective virus control seeing that essentially no significant difference in growth rate and then treated with the p53 the wild type p53 adenovirus we see essentially total cell death within three days in the cell line. This is not dependant upon this particular cell line. These are mutated p53 cell lines. These are wild type p53 cell lines treated with wild-type p53 adenovirus. Here mock infection replication defective virus. Here the adenovirus p53 treatment independent of cell line and here even though wild type endogenous p53 the cells although they have a little bit delay in their cell death, the cell death occurred within 3-4 days following transient infection. In nonmalignant cells, we sought to determine what this infection would produce. In fibroblast cell lines it has been established in laboratories. Here we have the mock infection in white; in green the replication defective virus and here the p53 adenovirus infection. The growth rate remains unchanged. Fibroblasts have nearly 100% transduction efficiency as compared to the cell lines and they express these infections without any difficulty. We sought to determine what may be the potential mechanism of cell loss that we were exhibiting in our in vitro growth assays. Here, in a DNA fragmentation experiment we have fragments and one hundred base pair ladder here. Here is mock infection. These are 24 hours after infection with the particular therapy. Mock infection a replication defective virus we see no evidence of any fragmentation and here in a time curve experiment have 4 hours, 8 hours, 22 hours you see the induction of fragmentation and at 30 hours. That is consistent with apoptosis death. Further studies are being done with regard to DNA labeling as well as electron microscopy in order to confirm this as the mechanism of in vitro as well as in vivo cell death that I'll show you in just a couple of minutes.

We then sought to determine in vivo model how we could develop novel molecular therapy in the management of this process. We have developed a flap model in a nude mouse. The flap model may use from one flap up to 4 flaps in these animals, depending on the particular experiments that I will describe to you later. A small incision is made in a flap that is elevated in

the subcutaneous plain and tumor cells are delivered into the flap and pipetted into the flap in order to deliver a particular number of tumor cells and also not to extravate the tumor cells outside of the pocket site. The tumor cells are held in place with a horizontal matra-suture that does not allow extravasations of any fluids that are delivered into the pocket. Then, the central therapeutic intervention is delivered. We have done experiments from 2 days later to up to 5 days later to deliver the novel therapy. In these earlier experiments, we did 3 flap models and in this flap we developed a flap, delivered the tumor, and then waited 3 or 4 days later in order to establish a subcutaneous nodule tumor. At that time, in these experiments, these were three days following this tumor, we elevated up the flap, isolated the nodule and then delivered the potential therapy. In the anterior flap here we delivered p53 adenovirus vector. In the posterior flap here we delivered replication defective virus. In the left posterior flank we delivered mock infection with phosphate buffered saline. We then allowed these animals to grow and allowed the tumor sites to grow. And as you see significant inhibition of tumor cell growth as compared to replication defective virus in the mock infection control. In a graphic sense, here in table format, the replication defective virus here has transport media alone which was PBS and the p53 adenovirus treated here and we see significant suppression as compared to replication defective virus through transport media alone independent of cell line utilized. And in these animals a slower growth curve. Two of these 6 animals had total suppression or total regression of the tumor both clinically as well as histologically when the flap model was evaluated histopathologically.

I enjoy Gary Larson and his depiction of potential laboratory peer pressure. One of the clinical dilemmas that we occur with in head and neck cancer is patients frequently develop or present with advance stage disease and one of the most frequently presented signs as well as symptoms is the development of the neck mass. Patients who develop the neck mass that is clinically palpable or greater than 2 cm in size, greater than 50% of them will have microscopic residual disease after aggressive surgical management of this process. To show you management of a process as depicted in the previous slide, here are a modified neck dissection is performed with the removal of the fibrolymphatic and facial contents of the superficial and deep compartments of the neck, major vascular, neural and muscular structures are spared from microscopic residual disease remains within this neck in the majority of our patients. In contemporary medicine today management of microscopic residual disease is performed with external beam radiation therapy. The results I've already described to you with advance stage disease. We continue to have major control problems in local and regional disease. This is a potential model for the delivery of novel intervention.

At this time, the entire environment for microscopic residual disease is available for intervention. How can we potentially predict those patients that have microscopic residual disease? We know that patients that develop neck masses greater than 2 cm in size, greater than 50% of these patients by the work of Barnes et al. of the University of Pittsburgh, have extracapsular or microscopic residual disease in the neck. The more radical the surgery does not change that process. As this size increases, the percentage approaches nearly 100%. Patients that present with neurotropic symptoms of their cancers, for example, diskinesia or akinesia of the cranial nerve or paralysis of the cranial nerve almost 100% of them will have microscopic residual disease that will require additional therapy in order to manage their process. Patients of the head and neck have a particular process that may induce field carcinogenesis that has been

proposed and propagated by many investigators within and outside of this institution. Those of our patients that have diffuse mucosal diseases that have erythroleukoplatic changes throughout their digestive tract, we frequently are fraught with chasing these margins from a surgical as well as from other intervention standpoints. Those patients that have deeply infiltrated tumors frequently have microscopic residual disease also.

We sought to develop a model for microscopic residual disease and novel intervention in this type of a model. In this model, we sought to determine transduction efficiency in our flap model that I previously described for you. In this model, we elevate the flap as I described to you before and then with our intervention I delivered mock infection or here the  $\beta$ -galactosidase adenovirus expression vector to determine simply transduction efficiency. Here mock infection we see no evidence of any ex-gal expression. Here with  $10^6$  plaque forming units being delivered into this environment we see a small amount of expression of the  $\beta$ -galactosidase. Here at  $10^7$  there essentially a log phase increase viral dose. We see more diffuse expression of the  $\beta$ -galactosidase and some inflammatory or edematous changes here. And here at 1 log dose even higher diffuse expression throughout multiple layers including the subdermal as well as muscular layer. Again, some edematous changes are also seen in this pocket that may be result of viral toxicity.

Utilizing the adenovirus p53 vector we then sought to establish small tumors within the pockets and then deliver the adenovirus vector. These are microscopic tumors. They're not clinically palpable at the time of intervention. This is a wild type tumor cell line that we utilize in order to make this more demonstrable for you today. Here to orient you is the dermal components and then the underlying tissues. The tumor here is established. This is treated with mock infection with phosphate buffered saline and then this is immunostained with p53 antibody that will detect both mutated and wild type p53. Here no significant expression of p53 protein. Here again to orient you, the overlying skin, the pocket of tumor, here treated with  $10^6$  plaque forming units of the wild type p53 adenovirus we see expression of the p53 proteins within the tumor. We see some peripheral necrosis but you certainly do see some viable tumor there in this pocket. At one log dose higher here we see the overlying skin. Here we see diffuse expression of the p53 protein from the muscular layer beneath to the subdermal area there is no evidence of any viable tumor and here with our highest viral dosing at  $10^9$  plaque forming units the overlying skin again to orient you some artifact here on sectioning but we see total vacuolization of the tumor. We see diffuse expression of the p53 protein and we see some inflammatory cells within the surrounding areas. Probably as a result of viral toxicity.

What happens if we allow these animals to continue to grow? Here in animal experiments and clearly here is the wild type p53 adenovirus vector treatment site replication defective virus and here control phosphate buffered saline alone in these animals treated with  $10^8$  plaque forming units, no tumors developed. Here the respective control sites. These animals will essentially have to be sacrificed because of outgrowth of this, no tumor has developed. Dose response experiments were done with animals in the four flap models that I showed you before as well as individual animals. I used the individual animals here in order to depict this for you. Here at  $10^9$  plaque forming units for the tumor burden of  $2.5 \times 10^6$  cells no tumor will ever develop.  $10^8$  plaque forming unit no tumor will ever develop in this particular cell line. At  $10^7$

plaque forming unit a small tumor will develop and will eventually outgrow the animal will succumb to this and here with a mock infection control the tumor growth.

In a table format we did multiple experiments. Both of these are mutated p53 cell lines. These are wild type p53 cell lines and here what we see with the microscopic residual disease model treated with  $2 \frac{1}{2}$  million cells that those mutated cell lines, none of them will ever develop tumors treated with  $10^8$  plaque forming units and this particular wild type p53 parental cell line none will develop tumors, but in this particular cell line it also has HPV positive, 2 of the 6 will develop tumors treated with  $10^8$  plaque forming units at  $10^9$  plaque forming units we have never seen tumor outgrowth. These results are very intriguing and exciting to us and therefore sought to determine what effects they may have on markedly gross residual disease. In these experiments we established subcutaneous nodules within these animals and then allowed these nodules to grow to a tumor burden of greater than one cm. In this experiment we used the  $\beta$ -galactosidase adenovirus vector in order to establish transduction efficiency in a large established tumor. This tumor was treated with a single injection of  $\beta$ -galactosidase adenovirus in 100 microliter aliquot. It was injected and this is the artifact or the vacuolization that occurred in the area of the injection, but their diffuse expression of the  $\beta$ -galactosidase adenovirus within this established tumor. This is not limited to a single cell layer. For your orientation, this is a 63 fold magnification. Please ignore the anterior component of these animals, but in the posterior flanks these were treated with replication defective virus. These were treated with the wild type p53 adenovirus. We treated them with a schema for gross tumor. In this schema these  $10^9$  plaque forming units on a three times weekly basis for two consecutive weeks. **Following two consecutive weeks in every animal tested, we had greater than 50% reduction in the tumors.** There is some overlying dermal loss here with ulceration here with the replication defective virus in both of these two sites this also occurred.

To summarize our investigation to this point in time, we are excited about the results of p53 adenovirus as it effectively inhibits the implanted tumors of squamous cell carcinoma, cell lines of the head and neck. This happens in vitro as well as in vivo. Tumor growth in established tumors is inhibited in a dose dependant fashion. We can titrate this upon the number of tumor cells that we implant into the model. Basically, we know that our squamous cell head and neck tumor cell lines will acquire approximately 1 million cells in order to grow in nude mice. We can give them tumor burdens up to  $5 \times 10^6$  cells or five million cells and effectively inhibit these with adequate transduction with p53 adenovirus. In time course experiments we know that the intervention needs to be established before there is gross palpable disease otherwise repeat treatment is required. **p53 adenovirus is effective in growth inhibition in squamous cancers of the head and neck in these in vitro as well as in vivo animal models independent of the parental cell lines p53 status.**

I have to tell you that this is unique to the squamous cell cancer of the head and neck. In Dr. Roth and Wei-Wei Zhang's work, this has not been shown in the non-small cell lung cancer model. What is indigenous to squamous cell cancer to the head and neck that makes these transformed cells responsive to this particular therapy unlike other solid organ tissue systems that have been established have not been clearly elucidated at this point in time. The mechanism of the induction of cell death by the wild type p53 adenovirus appears to be by the induction of apoptosis by our DNA fragmentation experiments. We are presently collaborating with Dr.

McKay of this institution with electron microscopy in order to determine whether the pathoneumonic findings of apoptosis are being induced both in vivo as well as in vitro equally DNA and leveling experiments are also being performed. Importantly transduced nonmalignant cells did not exhibit the same effect as we saw as the transformed squamous cell cancers of the head and neck. This is important number 1 because there is bystander transduction that we have shown in ours as well as other investigators have shown. This transduction is involved with a replication defective virus vector and therefore permanent integration is not foreseen has a problem as compared to other potential vectors for example, retroviruses, where permanent integration in nontransformed cells may be a significant potential dilemma.

I'd like to acknowledge several people and you never can thank enough people. I have clinical and scientific mentors galore in this institution. I have a department chairman named Helmut Gefford who is immensely supportive of all of our research efforts. He is immensely supportive both clinically and from a research standpoint and none of this could be accomplished without his support. Our laboratory works tirelessly. I don't like Dr. Gefford talking about how hard I work without acknowledging all these other people. Dr. Liu, our research associate and instructor within our laboratory is here night and day and on weekends. Dorothy Taylor our laboratory coordinator has done the majority of our drug curve experiments. Xiu-Wei as well as Francois Janot - Dr. Janot was a visiting scientist here from the Gustav-Russi Institute and assisted us in these studies. I have had too numerous of scientific mentors to bring up at this time. Dr. Roth has been immensely supportive of these efforts and has generously contributed to these initial experiments and also contributed the virus that both me and Dr. Zhang have produced. Dr. Nicolson and this entire department of tumor biology have been supportive of me since day one. Doors are always open in this institution and the cross-talk that occurs between the clinician and the scientist makes this institution unique, unlike any other I think that's available. And it's an honor to be able to practice both medicine and do scientific research in this facility.

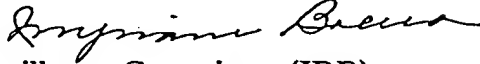
The University of Texas  
M. D. ANDERSON  
CANCER CENTER

MEMORANDUM

DATE:

\*

TO: Gary L. Clayman, DDS, M.D.  
Department of Head and Neck Surgery

FROM: Myriam Brena   
Secretary, Surveillance Committee (IRB)  
Office of the Vice President for Research

SUBJECT: Administrative Approval of Protocol HNS 94-001, entitled "Clinical Protocol for Modification of Tumor Suppressor Gene Expression in Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus Vector Expressing Wildtype p53"

Official Approval Date: \*

Dr. Aman U. Buzdar, Surveillance Committee Chairman, reviewed and administratively approved the above named and numbered protocol. Approval needs ratification at the next IRB meeting which will be held

\*

It was noted that the protocol and informed consent document are satisfactory and in compliance with federal and institutional guidelines.

Patients may not be entered on this protocol until it has been officially activated by the Office of Protocol Research.

In keeping with the requirements of the Department of Health and Human Services and the Food and Drug Administration, this clinical study must be reviewed twelve months from the date of approval. If the study is terminated or completed during the next twelve months, the Surveillance Committee should be so advised. You are responsible for promptly reporting to the Surveillance Committee:

- a) any severe adverse effects;
- b) any death while patient is on study;
- c) any unanticipated problems involving risks to subjects or others;
- d) any proposed changes in the research activity (changes may not be initiated without Surveillance Committee review and approval, except where necessary to eliminate apparent immediate hazards to the subjects).

c: Michael J. Keating, M.D.



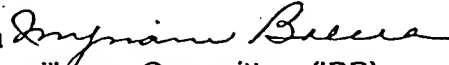
The University of Texas  
M. D. ANDERSON  
CANCER CENTER

MEMORANDUM

DATE:

\*

TO: Dr. Michael J. Keating  
Office of Protocol Research

FROM: Myriam Brena   
Secretary, Surveillance Committee (IRB)  
Office of the Vice President for Research

SUBJECT: Administrative Approval of Protocol HNS 94-001, entitled "Clinical Protocol for Modification of Tumor Suppressor Gene Expression in Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus Vector Expressing Wildtype p53"

Dr. Buzdar administratively approved the subject protocol and the pertaining memo is attached. However, this protocol cannot be activated until the Surveillance Committee reviews and approves it.

Thank you for your attention to this matter.

c: Gary Clayman, DDS, M.D.

THE UNIVERSITY OF TEXAS  
M. D. ANDERSON  
CANCER CENTER

\*

M E M O R A N D U M

TO: Dr. Gary L. Clayman  
Department of Head and Neck Surgery

FROM: Dr. Michael Tainsky *M Tainsky*  
Vice Chairperson, Institutional Biosafety Committee

SUBJECT: Microbial Document dated \*  
Agent: recombinant, adenovirus type 5, CMV wildtype p53  
(AdCMV p53)

The Institutional Biosafety Committee has approved the above document.

Thank you.

MT:blr  
cc: Dr. Jeffrey Tarrand

THE UNIVERSITY OF TEXAS  
M. D. ANDERSON  
CANCER CENTER

\*

M E M O R A N D U M

TO: Dr. Gary L. Clayman  
Department of Head and Neck Surgery

FROM: Michael Tainsky, Ph.D. *M Tainsky*  
Vice Chairperson, Institutional Biosafety Committee

SUBJECT: Approval of rDNA Registration Dated \*  
Entitled: Modification of Tumor Suppressive Gene  
Expression in Head and Neck Squamous Cell Carcinomas  
(HNSCC) with an Adenovirus Vector Expressing Wild-Type  
P53

The Biosafety Committee has approved the above document.

The Biosafety Committee has the responsibility of insuring compliance with regulatory agencies, such as the Recombinant DNA Advisory Committee (RAC). Before submission to RAC or any other agency, human subjects protocols and subsequent revisions pertaining to the use of recombinant DNA must receive full Biosafety Committee approval. After Surveillance Committee approval, please provide the Biosafety Committee secretary with eight copies of the human subject protocol and all correspondence received from the Surveillance Committee, RAC or any other agency.

Thank you.

MT:blr

cc:Dr. M. Frazier  
Dr. R. Legerski

THE UNIVERSITY OF TEXAS  
M. D. ANDERSON  
CANCER CENTER

\*

M E M O R A N D U M

TO: Dr. Gary L. Clayman  
Department of Head and Neck Surgery

FROM: Dr. Michael Tainsky *M. Tainsky*  
Vice Chairperson, Institutional Biosafety Committee

SUBJECT: Microbial Document dated \*  
Agent: recombinant, adenovirus type 5, CMV wildtype p53  
(AdCMV p53)

The Institutional Biosafety Committee has approved the above document.

Thank you.

MT:blr  
cc: Dr. Jeffrey Tarrand

#01

THE UNIVERSITY OF TEXAS  
M. D. ANDERSON CANCER CENTER

Protocol Title: Clinical Protocol for Modification of Tumor Suppressor Gene Expression in Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus Vector Expressing Wild-type p53

1. Participant's Name      I.D. Number

You have the right to know about the procedures that are to be used in your participation in clinical research so as to afford you an opportunity to make the decision whether or not to undergo the procedure after knowing the risks and hazards involved. This disclosure is not meant to frighten or alarm you; it is simply an effort to make you better informed so that you may give or withhold your consent to participate in clinical research. This informed consent does not supersede other consents you may have signed in other protocols.

**DESCRIPTION OF RESEARCH**

2. **PURPOSE OF THE STUDY:** Some cancers that occur in the head and neck area may be due to a defect in a gene called p53. The purpose of this clinical research study will be to see whether a normal copy of the p53 gene can be placed inside the patient's cancer cells and cause the cancer to grow more slowly or to stop growing.
3. **DESCRIPTION OF RESEARCH:** To introduce the normal p53 gene into tumor cells utilizing a defective virus of the adenovirus type which is similar to other viruses that cause the common cold. This defective virus is called a "vector". These tumors will be injected directly with the adenovirus three times a week for two weeks. After two weeks of rest from receiving treatment, tumors will then be injected again with the virus three times a week for two consecutive weeks. If the tumor can be removed in its entirety, surgery will be performed for removal of tumor that is considered completely removable by the patient's physician. Surgery must be performed within four days after completion of the last injection of virus. At the time of surgery, additional virus is placed into the area where the tumor has been removed. Tubing, which is ordinarily placed in surgical areas, is used to allow for drainage of fluids from the surgery area. Three days after the tumor has been removed, virus will be placed into the tubing and allowed to enter the surgical site once again. If the patient has undergone surgery, this will be the last treatment with adenovirus.

If the cancer cannot be removed by the physician, this tumor will be injected directly

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. Date of Activation.

. Signature J. B. Brea

three times a week for two consecutive weeks. After two weeks of rest from treatment, courses of three injections weekly for two consecutive weeks are repeated on a monthly basis. Injections of adenovirus are continued if tumor continues to shrink. If there is absence of tumor shrinkage, evidence of tumor growth, or adverse reactions to the adenovirus injections, treatment will be terminated.

The injections into the tumor are delivered with a skinny needle. If the tumor is greater than approximately two inches in size, approximately two teaspoons of virus will be injected into the tumor. Smaller tumors, less than two inches, will be injected with less than one teaspoon of virus. Multiple injections of virus into the tumors will be required with these injections being spaced apart by approximately one-half inch each. Numbing medication may be placed on the overlying skin to decrease discomfort from injections.

Patients with tumors of the throat or the voice box may also receive the treatment. It may be necessary to remove a part of the tumor surgically or with a laser before the treatment with p53 is given. Other patients may require that a tracheostomy is first performed. A tracheostomy is a surgical operation to make an opening into the windpipe. Certain routine diagnostic studies will be performed before entry into this trial. These involve local examination of the tumor by inserting an instrument with a light into the throat.

If previous specimens are insufficient for laboratory studies related to this research, additional biopsies will be needed. The treatment will be repeated monthly as long as there is evidence that the tumor is not growing.

The experimental treatment and costs related to the patient's participation in this research and which include clinical examinations, biopsies, and other forms of testing will be provided free to the patient. A maximum of 42 patients will be entered in this study. Twenty one patients may have injections of adenovirus with surgery and twenty one patients may have injections of adenovirus without surgery.

The patient's course will be followed indefinitely. Dr. Clayman's office should be notified if an address change is made.

**PERMISSION FOR AUTOPSY:** In case of death, the family of the patient will be asked for permission to perform an autopsy.

#### 4. RISKS, SIDE EFFECTS AND DISCOMFORTS TO PARTICIPANTS:

Two small additional biopsies will be required in addition to the initial biopsy. Risks from biopsies include coughing up blood which is usually slight. Severe hemorrhage which requires emergency treatment is rare. Biopsy of neck masses may also be associated with a slight risk of bleeding or infection. This clinical research study may involve unforeseeable risks to the participant.

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• Signature Dr. Clayman

*Possibility of Causing a New Cancer.* It is possible that the research could cause cancer in normal cells although this risk is believed to be small when the injected virus has been properly safety tested prior to its use. The adenovirus vector has only been used, to date, on the lining of the breathing tube in over twenty patients with no ill effects noted.

- 4a. This clinical procedure may involve unforeseeable risks to the unborn children, therefore, the participants should practice adequate methods of birth control throughout the period of their involvement in the clinical study if they are sexually active. To help prevent injury to children, the female participants should refrain from breast feeding during participation in the clinical research study.

5. **POTENTIAL BENEFITS:**

This treatment may result in shrinkage of the tumor, which may decrease cancer associated symptoms or may prolong life.

6. **ALTERNATE PROCEDURES OR TREATMENTS:**

Chemotherapy or other experimental drugs may be an alternative for some individuals. These treatments cause shrinkage of cancer in a number of patients. Another option is to only control the symptoms of disease and not take chemotherapy treatment.

**UNDERSTANDING OF PARTICIPANTS**

7. I have been given an opportunity to ask any questions concerning the procedure involved and the investigator has been willing to reply to my inquiries. This procedure will be administered under the above numbered, title, and described clinical research protocol at this institution. I hereby authorize Dr. the attending physician/investigator and designated associates, to administer this procedure.
8. I have been told and understand that my participation in this clinical research study is voluntary. I may decide not to participate, or withdraw my consent and discontinue my participation at any time. Such action will be without prejudice and there shall be no penalty or loss of benefits to which I may otherwise be entitled, and I will continue to receive treatment by my physician at this institution.

Should I decide not to participate or withdraw my consent from participation in this clinical research, I have been advised that I should discuss the consequences or

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effects of my decision with my physician.

In addition, I understand that the investigator may discontinue the clinical research study if, in the sole opinion and discretion of the investigator, the study or treatment offers me little or no future benefit, or the supply of medication ceases to be available or other causes prevent continuation of the clinical research study. The investigator will notify me should such circumstances arise and my physician will advise me about available treatments which may be of benefit at that time.

I will be informed of any new findings developed during the course of this clinical research study which might be relevant to my willingness to continue participation in the study.

9. I have been assured that confidentiality will be preserved except that qualified monitors from the Food and Drug Administration, Microbiological Associates, Magenta Corporation (manufacturers of the virus), and National Cancer Institute may review my records where appropriate and necessary. Qualified monitors shall include assignees authorized by the Surveillance Committee of this institution provided that confidentiality is assured and preserved. My name will not be revealed in any reports or publications resulting from this study, without my expressed consent.
10. I have been informed that, should I suffer any injury as a result of participation in this research activity, reasonable medical facilities are available for treatment at this institution. I understand, however, that I cannot expect to receive any credit or reimbursement for expenses from this institution or any financial compensation from this institution for such injury.
11. I have been informed that I should inquire of the attending physician whether or not there are any services, investigational agents or devices, and/or medications being offered by the sponsor of this clinical research project at a reduced cost or without cost.  
  
Costs related to my medical care will be covered by the Introgen Sponsored Research Agreement. Clarification of specific cost issues may be addressed in section 3 of this informed consent. I have been given the opportunity to discuss the expenses or costs associated with my participation in this research activity.
12. It is possible that this research project will result in the development of beneficial treatments, devices, new drugs, or possible patentable procedures, in which event I understand that I cannot expect to receive any compensation or benefits from the subsequent use of information acquired and developed through my participation in this research project.
13. I understand that refraining from breast feeding and practicing effective contraception is medically necessary and a prerequisite for my participation in this clinical research

• IRB Approved Consent

• Date of Activation

• Signature [Signature]



14. I may discuss any questions or problems during or after this study with Dr. Gary L. Clayman at \_\_\_\_\_ In addition, I may discuss any problems I may have or any questions regarding my rights during or after this study with the Chairman or the Surveillance Committee at \_\_\_\_\_ I may in the event any problem arises during this clinical research contact the parties named above.

Based upon the above, I consent to participate in the research and have received a copy of the consent form.

~~WITNESS OTHER THAN PHYSICIAN  
OR INVESTIGATOR~~

SIGNATURE OF PARTICIPANT

**SIGNATURE OF PERSON  
RESPONSIBLE & RELATIONSHIP**

I have discussed this clinical research study with the Participant and/or his or her authorized representative using a language which is understandable and appropriate. I believe that I have fully informed this participant of the nature of this study and its possible benefits and risks, and I believe the participant understood this explanation.

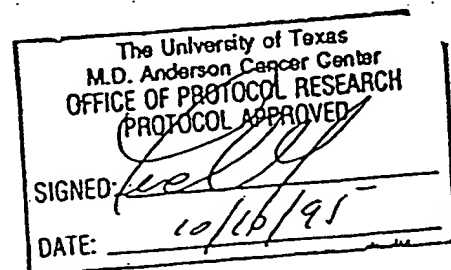
PHYSICIAN/INVESTIGATOR

- IRB Approved Consent
- Date of Activation: \*
- Signature *[Signature]*

THE UNIVERSITY OF TEXAS  
M. D. ANDERSON CANCER CENTER

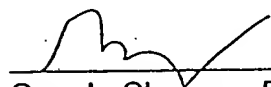
Protocol Title: *Clinical Protocol for Modification of Tumor Suppressor Gene Expression  
in Head and Neck Squamous Cell Carcinoma (HNSCC) with an  
Adenovirus Vector Expressing Wildtype p53*

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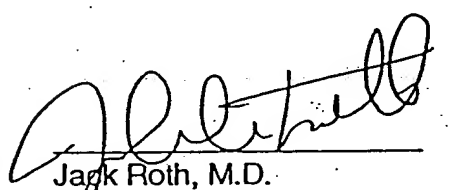


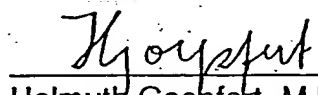
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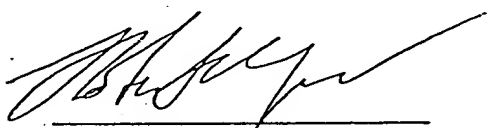
STUDY CHAIRMAN:

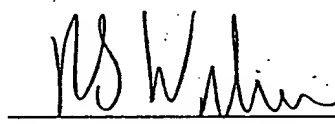
  
\_\_\_\_\_  
Gary L. Clayman, D.D.S., M.D.  
Department of Head and Neck Surgery

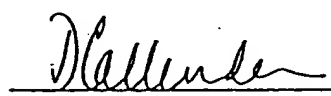
**STUDY COLLABORATORS:** Departments of Head and Neck Surgery; Thoracic and Cardiovascular Surgery; Thoracic, Head and Neck Medical Oncology; Pathology and Radiation Therapy.


  
\_\_\_\_\_  
Jack Roth, M.D.  
Department of Thoracic and Cardiovascular Surgery

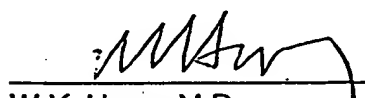
  
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Helmuth Goepfert, M.D.  
Department of Head & Neck Surgery

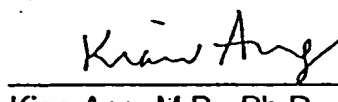
  
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Robert Byers, M.D.  
Department of Head & Neck Surgery

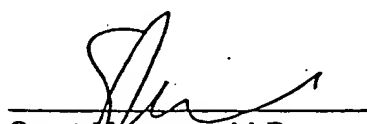
  
\_\_\_\_\_  
Randal Weber, M.D.  
Department of Head & Neck Surgery

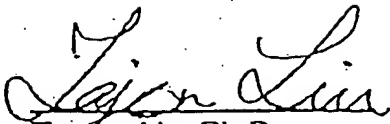
  
\_\_\_\_\_  
David Callender, M.D.  
Department of Head & Neck Surgery

  
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John Austin, M.D.  
Department of Head & Neck Surgery

  
\_\_\_\_\_  
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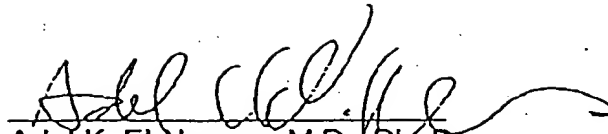
  
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## PROTOCOL ABSTRACT

Protocol: (Give number and abbreviated title)

(Two lines not to exceed 75 characters per line)

Clinical Protocol for Modification of Tumor Suppressor Gene Expression and Induction of Apoptosis in Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus Vector Expressing Wildtype p53

Study Chairman:

Gary L. Clayman, D.D.S., M.D., Department of Head and Neck Surgery

Patient Eligibility:

(Twenty lines not to exceed 75 characters per line)

1. Patients must have histologic proof of squamous cell carcinoma of the head and neck. Patients must be either unable to receive conventional treatment (e.g. the patient received radiation therapy with or without surgery) or have failed conventional treatment. Those patients with extensive local or regional disease that have persisted or recurred following radiation therapy (with or without chemotherapy or surgery) and have clinically resectable, but likely non-curable (<10% disease free survival) are also eligible. Patients need not have received a trial of chemotherapy prior to entering this protocol. All eligible patients will be discussed at the Head and Neck Surgery Multidisciplinary Treatment Planning Conference prior to protocol enlistment.
2. Patients must have clinical evidence of advanced local and/or regional cancer which is unresectable or for which no meaningful resection with surgical margins will be obtainable.
3. All patients must have a life expectancy of at least 12 weeks and must have a performance status of  $\leq 2$  (Zubrod scale, Appendix B).
4. All patients must sign an informed consent indicating that they are aware of the investigational nature of this study in keeping with the policies of the hospital. The only acceptable form is the one attached at the end of this protocol.
5. Patients will be tested for HIV prior to entry onto the protocol and must be HIV-negative. Patients with upper respiratory infections will not be treated until the infection resolves.
6. Patients must have adequate bone marrow function (defined as peripheral absolute granulocyte count of  $>2,000/\text{mm}^3$  and platelet count of  $100,000/\text{mm}^3$ ), adequate liver function (bilirubin  $\leq 1.5 \text{ mg/dl}$ ), and adequate renal function (creatinine  $<1.5 \text{ mg/dl}$ ).

Treatment Plan: (Include dose adjustment)

(Twenty lines not to exceed 75 characters per line)

1. The study will be an open-label upward dose ranging study for adenovirus-p53 vector (Ad5CMV-p53) in two patient groups. The two groups of patients will consist of a) resectable and b) non-resectable recurrent disease. The first phase of the study will allow assessment of toxicities related only to the vector. Patients will receive an intratumor injection of Ad5CMV-p53. The initial dose will be  $10^6$  plaque forming units (PFU).
2. Dose Escalation: The adenovirus dose will increase in one  $\log_{10}$  increments for each group until  $10^9$  PFUs are reached. Patients entered at a given dose level will not be eligible for dose escalation. We estimate based on the achievable titers of adenovirus that six dose levels can be done thus requiring 21 patients in each group (resectable and non-resectable).
3. All patients shall be registered with the Data Management Office.
4. Patients with local-regional tumors will have injection of a total dose of 10 ml for tumors  $\geq 4 \text{ cm}$  in diameter or 3 ml for tumor  $<4 \text{ cm}$  in diameter of the adenovirus preparation with the appropriate number of viral particles at multiple sites percutaneously or transorally. The treatment will be repeated three times weekly for two weeks. Dose escalation may proceed after a minimum two week follow-up of the last patient entered into the previous dose level. Treatment will continue on a monthly basis as long as there is no tumor progression. After one year the patients will be evaluated for continuation of therapy.
5. Those patients with surgically resectable disease will be treated by tumoral injection of adenovirus preparation as described above. The treatment will be repeated for two consecutive courses. Within four days of completion of the second course, the patients will be eligible to proceed with surgical resection. Prior to surgical closure, 10 ml of adenovirus preparation will be administered into the surgical defect (operative bed) and allowed to remain in contact for 60 minutes. The wounds are then closed and drains placed.

**Patient Evaluation:**

(Twenty lines not to exceed 75 characters per line)

1. A complete history and physical to include performance status, recent weight loss, usual weight and concurrent non-malignant disease and its therapy, and all prior anticancer treatments must be recorded. History and physical will be performed prior to each course.
2. Laboratory studies shall include quantitative immunoglobulins; a CBC with differential and platelet count; SMA-12 and electrolytes, including creatinine, bilirubin, SGPT, alkaline phosphatase, HIV analysis, urinalysis, chest x-ray, pre and post-treatment.
3. All relevant information regarding viral dosage, tumor response, laboratory examinations, and treatment-related toxicities must be recorded before each treatment is given.
4. Core biopsies or Incisional biopsies will be obtained of accessible local and or regional tumor. Tumor specimens will be collected 72 hrs. after the last injection of the adenovirus during the first treatment cycle.
5. Biopsies will be analyzed for incorporation of the transduced gene into the host genomic DNA and expression of the transduced gene at the RNA level by standard hybridization techniques following polymerase chain reaction and by in situ hybridization.
6. All patients will be evaluable for response and toxicity following one course of therapy.
7. A blood sample will be collected three times at one-half hour intervals following injection of the adenovirus. These samples will provide leukocytes to analyze for uptake of adenovirus DNA. Serum will be tested for antibodies to adenovirus proteins. Patients will be tested monthly during treatment, monthly for the first three months following completion of treatment, every three months for the remainder of the year following completion of treatment, and then at least yearly thereafter.
8. Normal tissue samples will be collected during the follow-up visits and endoscopies. These will include samples of non-malignant mucosa, leukocytes, and germ cells, if possible. These tissues will be analyzed for incorporation of the adenovirus.
9. A staging CT scan of the head and neck to evaluate local and regional disease will be obtained on an every three month basis during treatment.

**Miscellaneous Information:**

(Include any other information that you feel is pertinent to the study)

(Three lines not to exceed 75 characters per line)

**Statistical Considerations:**

(Twelve lines not to exceed 75 characters per line)

Three patients will be entered at each dose level with 6 patients entered at the maximum tolerated or maximum attainable dose (limitations imposed by production of the adenovirus). A maximum of 21 patients will be entered into each study group, for a total of 42 patients for the entire study.

**Objectives:**

(Twelve lines not to exceed 75 characters per line)

1. To determine the maximum tolerated dose of the wild-type p53 adenovirus vector in patients with refractory NSCC.
2. To determine the qualitative and quantitative toxicity and reversibility of toxicity of this treatment approach.
3. To document observed antitumor activity of this treatment approach.

Clinical Protocol: Modification of Tumor Suppressor  
Gene Expression in  
Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus  
Vector Expressing Wildtype p53"

1.0 OBJECTIVES

- 1.1 To determine the maximum tolerated dose of the wild-type p53 adenovirus vector in patients with refractory HNSCC.
- 1.2 To determine the qualitative and quantitative toxicity and reversibility of toxicity of this treatment approach.
- 1.3 To document observed antitumor activity of this treatment approach.

2.0 BACKGROUND AND RATIONALE

2.1 Molecular events in HNSCC

Head and Neck Squamous Cell Carcinoma accounts for nearly 45,000 thousand new cancers per year in the United States and in several parts of the world is one of the most frequent cancers. Nevertheless, mortality remains at nearly 55% and has not significantly changed since contemporary radiation therapy was implemented over thirty years ago. Patients with HNSCC are afflicted with a disease process which may have profound effects on speech, swallowing, cosmesis, and frequently cause social isolation. Unfortunately, all current treatment modalities, including radiation therapy, surgery, and chemotherapy, continue to have limited effectiveness in patients with advanced disease. Local/regional control remains a major issue in this patient population with only approximately 10% of patients with advanced disease succumbing to distant disease alone. The rational development of new therapies for HNSCC will depend on an understanding of the biology of this cancer at the molecular level. Molecular analysis has identified critical molecular events leading to HNSCC development and progression. The goal of this research is to directly modify the cancer cell to express large quantities of exogenously introduced a normal tumor suppressor gene product that suppresses the characteristics of the malignant phenotype.

The purpose of this protocol is to investigate molecular mechanisms that may influence the growth and progression of HNSCC; our goal is development of therapeutic agents specifically targeted at the molecular level. Approximately 80-85% of head and neck cancers are squamous cell carcinomas. Much of the current data on the molecular progression model of HNSCC has shown that multiple molecular events occur in the carcinogenic cascade. Although molecular studies have shown p53, int-2, Rb, Prad-1, and c-erb B2/Neu may all be involved in HNSCC, loss of heterozygosity microsatellite analysis has shown 3p, 9p, 14q, and 17p are frequent sites of loss (>40%) in adjacent premalignant areas.<sup>1</sup> The control and survival rates of patients with advanced head and neck cancers are low despite aggressive local regional therapy. Although dramatic responses have been observed with chemotherapy, its impact upon survival is minimal. Established biologic predictors of chemo or radiation therapy response have remained elusive. HNSCC also may serve as a model for other carcinogen-induced malignancies. The approaches and observations developed in this study may be applicable to other types of epithelial cancers.

Abundant evidence has accumulated that the process of malignant transformation is mediated by a genetic paradigm<sup>2</sup>. The major lesions detected in cancer cells occur in dominant oncogenes and tumor suppressor genes. Dominant oncogenes have alterations in a class of genes called proto-oncogenes, which participate in critical normal cell functions, including signal transduction and transcription. Primary modifications in the dominant oncogenes that confer the ability to transform include point mutations, translocations, rearrangements, and amplification. Tumor suppressor genes appear to require homozygous loss of function, by mutation, deletion, or a combination of these for transformation to occur. Some tumor suppressor genes appear to play a role in the governance of proliferation by regulation of transcription. It is possible that modification of the expression of

dominant tumor suppressor genes may influence certain characteristics of cells that contribute to the malignant phenotype.

Despite increasing knowledge of the mechanisms involved in oncogene-mediated transformation, little progress has occurred in developing therapeutic strategies that specifically target oncogenes and their products. Initially, research in this area was focused on dominant oncogenes, as these were the first to be characterized. DNA-mediated gene transfer studies showed acquisition of the malignant phenotype by normal cells following the transfer of DNA from malignant human tumors. Activated oncogenes of the *ras* family were identified by this technique with transfection of human DNA into mouse NIH 3T3 cells. More recently a class of tumor suppressor genes have been identified. Mutation or deletion of both copies of a tumor suppressor gene is required to eliminate its function and cause the cell to acquire characteristics of the malignant phenotype.

#### Tumor Suppressor Gene Mutations in Head and Neck Squamous Cell Carcinoma

The *p53* gene is the most frequently mutated gene yet identified in human cancers. It is mutated in over 50% of human HNSCC<sup>3</sup>. The *p53* gene encodes a 375-amino-acid phosphoprotein that can form complexes with viral proteins such as large-T antigen and E1B<sup>4</sup>. Missense mutations are common for the *p53* gene and are essential for the transforming ability of the oncogene. The wildtype *p53* gene may directly suppress uncontrolled cell growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wildtype *p53* may contribute to transformation. However, some studies indicate that the presence of mutant *p53* may be necessary for full expression of the transforming potential of the gene. Mutations of *p53* are common in a wide spectrum of tumors<sup>5-8</sup>; they occur in both HNSCC cell lines and fresh tumors<sup>9</sup>. Additionally, they occur in second primary cancers that may arise in over 20% of head and neck cancer patients.<sup>10</sup>

An option for specific targeting of tumor suppressor genes is replacement of a deleted or mutated tumor suppressor gene. Progress in the understanding of the critical genes involved in tumor development and in technology for altering gene expression logically led to our studies of techniques for achieving these options.

Our working hypothesis is that overexpression of a wildtype *p53* tumor suppressor gene in the cancer cell can potentially reverse critical features of the malignant phenotype of that cell. This finding has important therapeutic implications. Cancer cells have multiple genetic alterations. Therapy directed toward oncogenes will be practical only if therapeutic effects occur with targeting of one or two genes. It is unlikely that any therapy targeting oncogenes or their products will be absolutely specific for cancer cells. If other genes can compensate for loss of normal function by a specific oncogene mediated by an antisense construct, the harmful effects of the therapy will be reduced. Studies from Roth et al. indicate that reversal of a single genetic alteration has profound effects on the growth and tumorigenicity of carcinoma cells<sup>11</sup>. Additional support for this concept comes from a recent study by Soriano and co-workers<sup>12</sup> in which transgenic mice were created that lacked a functional *c-src* proto-oncogene. The resulting developmental defect in the mice was osteopetrosis. The ubiquity of *c-src*, its high degree of conservation among species, and its role in mitosis suggest that inactivation would be lethal, but this was not the case; viable mice were recovered. A possible explanation is that other closely related nonreceptor tyrosine kinases such as *yes* and *fyn* can compensate for loss of *c-src*. Introduction of a single copy of a wildtype tumor suppressor gene into normal cells would be unlikely to have adverse effects if it occurred during therapy directed at replacing inactivated tumor suppressor genes in cancer cells. To support this, work in our laboratory has investigated the effect of exogenous wild-type *p53* transduced in non-malignant fibroblasts and found that no alteration in cell growth or morphology occurs as compared to tumor cells that undergo cell cycle arrest, inhibition of tumorigenicity and apoptosis. (Clayman, unpublished data Appendix D. Figure 6.)

Wild-type *p53* appears dominant over its mutant gene and will select against proliferation when transfected into cells with the mutant gene<sup>11,13</sup>. Our experiments have shown that expression of the transfected *wtp53* does not affect the growth of non-malignant cells with endogenous *wtp53*. Thus, such constructs might be taken up by normal cells without adverse effects. This protocol will study



local or regional delivery of wtp53 to HNSCC patients with unresectable regionally metastatic squamous cell carcinomas, unresectable local cancers, or advanced local and/or regional HNSCC that have failed prior treatment which included radiation therapy but remains locally and regionally resectable. The efficiency of delivery and gene expression will be evaluated both in head and neck cancer cells and in normal cells *in vivo*. This is of importance for the design of constructs that may be useful therapeutically. The effects of these constructs on clinical progression of the cancer will also be studied.

These approaches may lead to cancer therapy based on direct alteration of gene expression in cancer cells. Current therapy relies on attempts to kill or remove the last cancer cell. However, tumor cell dormancy is an established phenomenon making effective killing highly unlikely. Although inhibition of expression of some oncogenes may be lethal to the cancer cell, in some cases cell replication will slow or cease, thus rendering these cancers clinically dormant. Nevertheless, the chosen delivery method of adenovirus vectors addresses this dormancy issue since transduction by the vector is cell cycle independent. Even if absolute specificity is not achieved, single oncogenes or tumor suppressor genes may still be important targets, because it is likely that adverse effects to normal cells will be minimal as well as transient with the transient adenovirus vector chosen.

Programmed cell death, also known as apoptosis, shows a characteristic pattern of DNA fragmentation resulting from cleavage of nuclear DNA and is considered to be a selective process of physiologic cell deletion. It has recently been reported that the wtp53 gene is involved in mediating programmed cell death of some types of tumor cells<sup>14-16</sup>. Our studies have shown that independent of the endogenous p53 status (homozygous mutated or wild-type), HNSCC cells transduced with recombinant adenovirus-mediated wtp53 gene exhibit histologic alterations both by light field and electron microscopy that are consistent with apoptosis. Similarly, *in vitro* infection with the same vector induces specific DNA fragmentation consistent with this mechanistic hypothesis. Nevertheless, this is in contrast to normal cells with wildtype p53 which are unaffected by wildtype p53. Direct injection of adenovirus p53 into subcutaneously established and microscopic HNSCC tumors in nu/nu mice induces apoptosis and dose response destruction and inhibition of tumor development and growth. (Clayman, unpublished data, Appendix D) These changes occur independent of the HNSCC tumor cell line endogenous p53 status (wildtype or mutated). These results support the use of this strategy in a clinical trial.

## 2.2 Natural history of locally unresectable HNSCC and M.D. Anderson Patient Patterns

Patients with HNSCC die of their cancer in approximately 55% of cases and failure of therapy at the primary or regional tumor site is a significant problem<sup>18,19</sup>. Of the 45,000 patients newly diagnosed with head and neck cancer in 1991, nearly half underwent surgical resection with or without radiation therapy. Local and/or regional recurrence as the first site of failure will occur in approximately 22,000 of all of those patients independent of the treatment modality chosen (surgery, radiation therapy, chemotherapy or combinations thereof). Thus, nearly 22,000 patients per year could benefit from improved local-regional therapy. Patients with local/regionally unresectable HNSCC, that has failed radiation therapy, have a median survival of approximately 6 months and no known systemic chemotherapy has shown significant survival benefit among these patients. The Department of Head and Neck Surgery at the University of Texas M. D. Anderson Cancer Center has extensive experience in the treatment of patients afflicted with squamous carcinoma of the upper aerodigestive tract. Over 1200 patients with head and neck cancer are seen yearly and over 600 of these patients undergo resections. From September 1992 to August 1993, 469 patients with local/regional squamous cell of the upper aerodigestive tract (no evidence of distant metastasis) were referred to the Department of Head and Neck Surgery at M. D. Anderson Cancer Center; 113 had prior radiation therapy (52 radiation therapy only, 40 surgery and radiation therapy, 13 chemotherapy and radiation therapy, and 8 chemotherapy + radiation therapy + surgery). Of these 113, 84 had local/regional squamous cell carcinoma without distant metastasis.

### 2.2.1 Measure of disease activity

The goal of this therapy is to halt or reverse the manifestations of the disease. The efficacy of

therapy in this group of patients will be measured by determining length of patient survival and reduction in measurable tumor mass. There is no curative therapy for this stage of disease and thus the outcome is predictable enough to allow for an assessment of the results of gene therapy. The measurements that will be used are described in Section 7.0.

#### 2.2.2 Anticipated effect of protocol treatment

It is anticipated that the administration of the adenovirus wildtype *p53* will decrease the rate of proliferation of these cells and induce apoptosis of infected malignant cells. This would reduce the growth rate or cause regression of primary and/or nodal disease and therefore relieve symptoms and potentially prolong the patient's survival.

#### 2.2.3 Alternative therapies

Patients with unresectable squamous cell carcinoma of the head and neck that have failed or are unable to receive external beam radiotherapy will be considered for this protocol. Existing therapies for this condition offers only the potential for short-term palliation. Most patients have recurred despite external beam radiotherapy. Patients receiving this treatment have a median survival of approximately 6 months. Patients failing brachytherapy would also be eligible to receive gene therapy. In those patients that may still have potentially resectable tumor, which has failed radiation therapy (alone or in combination with prior surgery or chemotherapy), in which tumor can be surgically excised following adenovirus *p53* gene therapy and then additional gene therapy can be delivered to the surgical bed after the tumor volume has been drastically reduced to microscopic disease will also be eligible. These patients, although technically resectable have the same prognosis as unresectable patients. Patients with unresectable local-regional tumors who have failed surgery or radiation therapy have a poor prognosis. Chemotherapy is only palliative and the median survival remains less than 6 months. The administration of the adenovirus constructs would not preclude the patient from receiving other palliative therapy if the tumor progresses.

### 2.3 Structure and characteristics of the biological system

#### 2.3.1 Restoration of expression of *wtp53* gene product

##### 2.3.1.1 Preliminary studies with plasmid DNA

The *p53* gene is the most commonly altered gene yet described in human cancers. To study this gene, a cell culture model system of cell lines varying in *p53* expression was established. The H322a adenocarcinoma cell line expresses the mutant *p53* protein as shown by the presence of high levels of endogenous *p53* mRNA and phosphorylated protein. We showed that the H322a cell line has a G:T transversion at codon 248 (Arg to Leu) with absence of the wildtype allele. The H322a cell line has a homozygous *p53* deletion. The H460a and H226b cell lines are homozygous for the wildtype *p53*. Expression vectors for sense (*S-p53*) and antisense *p53* (*AS-p53*) cDNA with a  $\beta$ -actin promoter were constructed to study the effect of *wtp53* expressed in lung cancer cells with mutant or deleted *p53* and the effects of reducing wildtype and mutant *p53* expression<sup>11</sup>.

Stable transfectants of *p53* mutant cells (H322a) or deleted *p53* (H358) expressing *S-p53* could not be rescued. Failure to isolate colonies expressing sense *p53* RNA in cells with homozygous mutant or deleted alleles shows that *wtp53* can suppress in vitro cancer cell growth in cells expressing a mutant *p53* or having a homozygous *p53* deletion.

In general, transfection with antisense *p53* (*AS-p53*) reduced colony formation (10-fold) by cells with endogenous mutant *p53*. This indicates that expression of mutant *p53* contributes to the transformed phenotype. As expected, cells with *wtp53*

(H226b) showed increased tumorigenicity when transfected with AS-p53. The H226b cells expressing AS-p53 grow significantly more rapidly in nu/nu mice than the cells transfected with the control plasmid. This indicates that elimination of the *wtp53* gene product enhances features of the malignant phenotype.

These studies showed that *wtp53* is dominant and can suppress the malignant phenotype in cells with mutant or deleted *p53*. The presence of the mutant *p53* confers transforming potential to the gene product, which can be suppressed by AS-p53. Thus, in cancer cells both the absence of *wtp53* and the presence of certain *p53* mutations may enhance the malignant phenotype.

#### 2.3.1.2 Generation of recombinant p53 adenovirus.

The p53 expression cassette (Figure 1, Appendix D), which contains human cytomegalovirus (CMV) promoter<sup>21</sup>, wild-type p53 cDNA, and SV40 early polyadenylation signal, was inserted between the Xba I and Cla I sites of pXCJL1, a plasmid kindly provided by Dr. Frank L. Graham of McMaster University, Hamilton, Ontario, Canada. The p53 shuttle vector (pEC53) and the recombinant plasmid pJM1722 were cotransfected into 293 cells<sup>23</sup> by liposome-mediated transfection with DOTAP (Boehringer Mannheim Corp., Indianapolis, IN)<sup>24</sup>. The transfected cells were maintained in medium until the onset of the cytopathic effect. Identification of newly generated p53 recombinant adenoviruses (Ad5CMV-p53) with PCR analysis of the DNA samples prepared from the cell culture supernatants was described elsewhere<sup>24</sup>. The wild-type sequence of the p53 cDNA in the Ad5CMV-p53 virus was confirmed by dideoxy DNA sequencing on the CsCl-gradient-purified viral DNA. The control virus Ad5/RSV/GL2, generated in a similar manner, has a structure similar to that of Ad5CMV-p53 except a Rous sarcoma viral promoter and luciferase cDNA were used in its expression cassette. The recombinant adenovirus that carries a *E. coli*  $\beta$ -galactosidase gene (LacZ), Ad5CMV-LacZ, also has a structure similar to that of Ad5CMV-p53, and was kindly provided by Dr. Frank L. Graham. Structural analysis of the vectors is shown in Fig. 2, Appendix D.

2.3.1.3 Viral stock, titer, and infection. Individual clones of the Ad5CMV-p53, Ad5/RSV/GL2, and Ad5CMV-LacZ viruses were obtained by plaque-purification according to the method of Graham and Prevec<sup>25</sup>. Single viral clones were propagated in 293 cells. The culture medium of the 293 cells showing the completed cytopathic effect was collected and centrifuged at 1000 x g for 10 min. The pooled supernatants were aliquoted and stored at -20°C as viral stocks. The viral titers were determined by plaque assays<sup>25</sup>. Infections of the cell lines were carried out by addition of the viral solutions (0.5 ml per 60-mm dish) to cell monolayers and incubation at room temperature for 30 min with brief agitation every 5 min. This was followed by the addition of culture medium and the return of the infected cells to the 37°C incubator.

#### 2.3.1.4 Preclinical studies

Expression of exogenous p53 protein in HNSCC cells. To obtain a high level expression of p53, the human CMV promoter<sup>21</sup> was used to drive the p53 cDNA carried by Ad5CMV-p53. As shown by immunostaining and Western blot in Figures 3 and 4 (Appendix D), a high level of expression of exogenous p53 was achieved in the HNSCC cell lines Tu138 and Tu177 that were infected by Ad5CMV-p53 at an MOI of 50 PFU/cell. When Tu138 or Tu177 cells were infected at the same MOI, the level of expression of the exogenous wild-type p53 gene was at least four times higher than that of the endogenous mutated protein in Tu138 and 14 times higher than that of the endogenous wild-type protein in MDA 1986 cells (data not shown). The time course of the expression of the exogenous p53 after a single infection of 10 PFU/cell was studied in H358 cells. The protein expression peaked at post-infection day 3, sharply decreased after day 5, and lasted for at least 15 days (Fig. 5, Appendix D).

PCR analysis on the DNA samples prepared from the Ad5CMV-p53-treated H358 cells failed to detect the viral DNA after post-infection day 15 (data not shown). The decrease in expression of the exogenous p53 probably resulted from the cellular attenuation on the CMV promoter or degradation of the viral DNA in the treated cell population<sup>26</sup>. This is a critical point with respect to safety of the vector. Transient p53 expression is sufficient for mediating apoptosis. However, normal cells taking up the vector will express the exogenous p53 for only a short time.

**Effect of exogenous p53 on HNSCC cell growth.** Five human HNSCC cell lines were chosen for this study: cell line Tu138 and Tu177, which both have homozygous mutations of the p53 gene, and cell lines MDA 686, 886 and 1986 which have wild-type p53 genes. Additionally, fibroblasts derived from stromal tissue culture outgrowth of patients afflicted with head and neck cancer were also assessed. The cells were treated with Ad5CMV-p53 and dl312 (replication defective adenovirus control) at 50 PFU/cell. Triplicate sets of the viral-infected and mock-infected cells were counted every day for 6 days. Growth rates of the Ad5CMV-p53-infected HNSCC cells were significantly inhibited compared to that of the mock-infected cells and dl312 infected cells. (Fig. 6., Appendix D). Unlike other epithelial cancer cell lines previously tested, within 72 hours post-infection, no viable cell colonies can be obtained in reculturing transduced cell culture dishes. Twenty-four hours after infection, an apparent morphologic change occurred with portions of the cell population rounding up and their outer membranes forming blebs. Cells infected with dl312, (replication-defective adenovirus), demonstrated normal growth characteristics with no histomorphologic abnormalities. Ad5CMV-p53 had no effect on cell growth or morphology in non-malignant fibroblasts derived from cancer patients. (Fig. 6, Appendix D)

**Inhibition of tumorigenicity mediated by Ad5CMV-p53.** To examine whether the Ad5CMV-p53 virus can inhibit tumorigenicity of human HNSCC cells, the effect of AdCMV-p53 on established subcutaneous tumor nodules was determined in nude mice in a defined pathogen-free environment. Briefly, following induction of acepromazine anesthesia, three separate s.c. flaps were elevated on each animal and  $5 \times 10^6$  cells in 150 $\mu$ l nude mice were injected s.c. After four days, palpable tumors were noted in each pocket site (6mm<sup>3</sup> or greater in size) and animals were reanesthetized, and the flaps were re-elevated for the delivery of 100 $\mu$ l of a) Ad5CMVp53 ( $10^8$  P.F.U.) in the right anterior flap; b) replication defective adenovirus, dl312, ( $10^8$  P.F.U.) in the right posterior flap and c) transport medium alone in the left posterior flap. Animals were observed daily and sacrificed on day 20 following experimental interventions.

Seven animals were tested for each cell line. One animal in the Tu-177 group died following the second flap surgery and delivery of the therapeutic interventions, presumably due to profound anesthesia and subsequent mutilation by cage mates. Necropsy revealed no evidence of metastasis or systemic effects. Figure 7, Appendix D shows representative Tu-138 (left) and Tu-177 recipients (right). Sizable tumors are apparent on both posterior flaps of the animals (i.e., the sites that did not receive Ad5CMV-p53). The lack of tumor progression is significant in the right anterior flaps of the animals, which received Ad5CMV-p53 in both cell lines tested. That Tu-177 cells have a slower growth rate has previously been established in these animals (unpublished data). Two animals in the Tu-177 group had complete clinical and pathologic regression of their established subcutaneous tumor nodule. Two animals in the Tu-138 group were killed early because they were experiencing rapid growth and ulceration of the control tumor sites. All surgical sites had developed lesions of at least 6 mm<sup>3</sup> before intervention. The tumor volumes on necropsy are shown in Table 1.

Table 1 Effect of Ad5CMV-p53 on Established tumor growth in nude mice<sup>a</sup>

Treatment	Mean volume (mm <sup>3</sup> ± SEM)	
	Tu-138 (7)	Tu-177 (6)
Ad5CMV-p53	22.3 ± 14	43 ± 18
Ad5(dl312)	803 ± 300	533 ± 148
Medium	1297 ± 511	421 ± 143
Significance	p value	p value
p53 <sup>b</sup> : dl312	0.03	0.02
p53 : Medium	0.04	0.03

<sup>a</sup>The cells were injected subcutaneously at 5 x 10<sup>6</sup> cells/flap. Tumor sizes were determined at day 20 after treatment. Numbers in parentheses represent the number of animals evaluated.

<sup>b</sup>Ad5CMVp53 is abbreviated as p53; dl312 is an abbreviation for Ad5(dl312). Statistical analysis by Friedman's two-way analysis of variance.

The efficacy of Ad5CMV-p53 in inhibiting tumorigenicity was further evaluated in the mouse model of microscopic residual disease in HNSCC. Representative HNSCC cell lines of homozygous mutations, as well as wild-type p53 cell lines were used. In head and neck cancer, as well as several other solid malignancies, direct gene transfer to microscopic residual carcinoma may not be so technically difficult. When the primary source of tumor is removed, the tumor base is readily available for molecular therapy as well as the most likely pathway of lymphatic spread when a neck lymph node dissection is performed. To investigate these issues, we designed our experiments to determine if in vivo adenovirus p53 mediated gene transfer would effect the establishment or growth of SCCHN cells implanted into a subcutaneous flap. Athymic nude female mice were anesthetized and three subcutaneous flaps elevated and the SCCHN cell lines pipetted subcutaneously in order to prevent erroneous tumor inoculation and dispense a specified number of cells. Following 48 hours, mice were reinoculated with either adenovirus p53, transport medium alone, and a non-marker replication defective adenovirus dl312 or the adenovirus β-galactosidase vector. The development of tumors was tumor cell number dependent, allotted time for implantation dependent, and dose dependent upon adenovirus p53. A representative viral dose response experiment is shown in figure 8, Appendix D. Dose response experiments with the marker Galactosidase gene clearly demonstrate dose-response transduction efficiency in this model (figure 8) and was also confirmed by p53 immunohistochemistry three days following infection (figure 9, Appendix D). In the presence of microscopically implanted tumor of 2.5-5.0 x 10<sup>6</sup> cells treated with adenovirus p53 at 10<sup>8</sup> P.F.U. or greater, tumors developed in 2 of 6 animals in only one of the wild-type cell lines (886LN). All other tumor cell line exhibited total inhibition of tumor development both grossly and histologically. These experiments clearly indicate that microscopic proliferating tumor cells can be successfully infected in vivo if exposed to adenovirus p53. Tumor formation was evaluated at the end of a 8-week period by gross and histologic analysis of the surgical sites. The data of tumor measurements are summarized in Table II. These results indicate that Ad5CMV-p53 can prevent the formation of HNSCC in a subcutaneous model of microscopic residual disease.

Table II. Effect of Ad5CMV-p53 on Tumorigenicity in a Microscopic Residual Disease Model of HNSCC.<sup>a</sup>

Cell line	Treatment		
	No. mice w/ Total Mice (%)		
	Vehicle (PBS)	dl312	Ad5CMV-p53
Tu138 (homozygous mutation p53)	8/8	8/8	0/8
Tu177 (homozygous mutation p53)	8/8	8/8	0/8
686 LN (homozygous wild-type p53)	5/8	5/8	0/8
886 (homozygous wild-type p53)	6/6	6/6	2/6

<sup>a</sup> Mice were inoculated with  $2.5 \times 10^6$  /flap subcutaneously. On the 2nd day post-inoculation, the mice were given either vehicle or viruses ( $1 \times 10^8$  P.F.U. each in 0.1 ml) in the same flap site in a single intervention strategy. Tumor formation was evaluated at the end of a 8-week period.

In a treatment strategy to determine the role of repeat treatment of AdCMVp53 in established tumors, subcutaneously established HNSCC tumor cell lines were peritumorally infiltrated with either vehicle (PBS) or viruses ( $1 \times 10^9$  P.F.U. each in 0.1 ml) three times weekly for two consecutive weeks. Tumor burdens exceeding 1 cm in greatest dimension were utilized in these studies. A greater than 50% reduction in Tu138 tumor mass was seen in 5/5 animals treated in this study with significant reduction in size compared to control-treated sites ( $p < 0.04$ ). (Figure 10, Appendix D) No evidence of systemic toxicity was clinically or histologically noted in whole organ necropsy studies. In additional repeat treatment studies using smaller established tumors that had reached 75mm<sup>3</sup>, were similarly peritumorally treated with identical controls as described above. In both Tu177 and Tu138, following 7 peritumoral AdCMVp53 treatments (3 times weekly), 8 of 9 animals had complete regression of disease whereas control animals showed continued progression of tumors as well as no evidence of systemic toxicity in every animal (10 of 10).

### 3.0 SAFETY INFORMATION

- 3.1 Continued absence of replication competent infectious virus was determined from sequential infection experiments. No replicative virus was detectable by PCR analysis of DNA samples from HeLa cells treated with the frozen/thawed cell extracts from HeLa cells initially infected with Ad5CMV-p53 at 100 PFU/cell. Ad5CMV-p53 was confirmed as a replication-defective and helper-independent virus. Further confirmation of this was obtained by labeling viral supernatants with [<sup>3</sup>H]Thymidine. Absence of labeling in extracted DNA showed absence of replication competent adenovirus. These studies and the following safety studies will be performed by Microbiological Associates, Inc.
- 3.2 Sterility will be assured by testing for aerobic and anaerobic bacteria, fungus, and mycoplasma. Other tests to be performed by Microbiological Associates, Rockville, MD include:

Transmission EM for Viruses  
*In Vitro* Assay for Adventitious Viral Contaminants  
*In Vivo* Assay for Adventitious Viral Contaminants  
Isoenzyme & Cytogenetic Analysis  
Tumorigenicity<sub>c</sub>  
EBV  
CMV  
Hepatitis  
HIV Co-Cultivation  
HTLV 1/2 PCR  
Adeno-Associated (AAV) Hybridization  
Parvovirus B-19 Hybridization Adenovirus

#### 4.0 PATIENT ELIGIBILITY

- 4.1 Patients must have ~~histologic proof of squamous cell carcinoma of the head and neck~~. Patients must be either ~~unable to receive conventional treatment (e.g. the patient received radiation therapy with or without surgery) or have failed conventional treatment~~. Those patients with extensive local or regional disease that have persisted or recurred following radiation therapy (with or without chemotherapy or surgery) and have clinically resectable, but likely non-curable (<10% disease free survival) are also eligible. Patients need not have received a trial of chemotherapy prior to entering this protocol. All eligible patients will be discussed at the Head and Neck Surgery Multidisciplinary Treatment Planning Conference prior to protocol enlistment.
- 4.2 ~~Patients must have clinical evidence of advanced local and/or regional cancer which is unresectable or for which no meaningful resection with surgical margins will be obtainable.~~
- 4.3 ~~All patients must have a life expectancy of at least 12 weeks and must have a performance status of  $\leq 2$  (Zubrod scale, Appendix B).~~
- 4.4 All patients must sign an informed consent indicating that they are aware of the investigational nature of this study in keeping with the policies of the hospital. The only acceptable form is the one attached at the end of this protocol.
- 4.5 Patients will be tested for HIV prior to entry onto the protocol and ~~must be HIV negative~~. Patients with upper respiratory infections will not be treated until the infection resolves.
- 4.6 Patients must have adequate bone marrow function (defined as peripheral absolute ~~granulocyte~~ count of  $\geq 2,000/mm^3$  and platelet count of  $\geq 100,000/mm^3$ ), adequate liver function (bilirubin  $\leq 1.5$  mg/dl), and adequate renal function (~~creatinine  $\leq 1.5$  mg/dl~~).
- 4.7 Female patients of child-bearing potential are excluded.

#### 5.0 TREATMENT PLAN

- 5.1 The study will be an open-label upward dose ranging study for adenovirus-p53 vector (Ad5CMV-p53).
- 5.2 The study will be done with two groups of patients. The two groups of patients will consist of a) resectable and b) non-resectable recurrent disease (Refer to 4.1). It is not known what toxicities if any will be caused by the adenovirus. The first phase of the study will allow assessment of toxicities related only to the vector. Patients will receive one intratumor injection of Ad5CMV-p53. The initial dose will be  $10^6$  plaque forming units (PFU).

- 5.3 Three patients will be entered at each dose level with 6 patients entered at the maximum tolerated or maximum attainable dose (limitation imposed by production of the adenovirus).
- 5.4 Dose Escalation: The adenovirus dose will increase in one-log<sub>10</sub> increments for each group until 10<sup>9</sup> PFUs are reached. Patients entered at a given dose level will not be eligible for dose escalation. We estimate based on the achievable titers of adenovirus that six dose levels can be done thus requiring 21 patients in each group (resectable and non-resectable).
- 5.5 All patients shall be registered with the Data Management office at \_\_\_\_\_ At the time of registration, a prestudy form shall be necessary on each patient. When applicable, information pertaining to important prognostic factors such as tumor histology, pretreatment weight loss, performance status, disease stage and extent, and prior therapy will be requested.
- 5.6 Patients with local-regional tumors will have injection of a total dose of 10 ml for tumors  $\geq 4$  cm in diameter or 3 ml for tumor  $< 4$  cm in diameter of the adenovirus preparation with the appropriate number of viral particles at multiple sites percutaneously or transorally. Direct endoscopic injections may also be used is required. Injections will be placed at approximately 1 centimeter increments.
- 5.7 The treatment will be repeated three times weekly for two weeks. Dose escalation may proceed after a minimum two week follow-up of the last patient entered into the previous dose level. Treatment will continue on a monthly basis as long as there is no tumor progression. After one year the patients will be evaluated for continuation of therapy.
- 5.8 Patients will wear a surgical mask for 24 hours following injection of the adenovirus. All medical personnel will wear masks and face shields routinely during endoscopy and injection of the adenovirus. Anti-tissves will be prescribed as necessary. All patients will be kept in isolation during the time they are receiving injections of the adenovirus and for 48 hours after the last injection.
- 5.9 Those patients with surgically resectable disease will be treated by tumoral injection of adenovirus preparation as described in 5.6 and 5.7. The treatment will be repeated for two consecutive courses. Within four days of completion of the second course, the patients will be eligible to proceed with surgical resection. At the completion of surgical resection, prior to closure, 10ml of adenovirus preparation will be administered into the surgical defect (operative bed) and allowed to remain in contact for 60 minutes. The wounds are then closed and drains placed. Post-operatively, on the third post-operative day (prior to drain removal), 10ml of adenovirus preparation is sterily introduced into the drains and retrograde placed into the wounds and is allowed to remain for two hours. The drains are then replaced to suction and removed when indicated by the attending staff surgeon.

## 6.0 PRE-TREATMENT EVALUATION

- 6.1 A complete history and physical to include performance status, recent weight loss, usual weight and concurrent non-malignant disease and its therapy, and all prior anticancer treatments must be recorded.
- 6.2 Laboratory studies shall include quantitative immunoglobulins; a CBC with differential and platelet count; SMA-12 and electrolytes, including creatinine, bilirubin, SGPT, alkaline phosphatase, HIV analysis, urinalysis, and chest x-ray.
- 6.3 Any residual toxicity from prior therapies should be recorded using the grading schema in Appendix C.
- 6.4 Appropriate studies should be obtained to fully define the extent and severity of existing or suspected malignant and non-malignant disease.
- 6.5 Measurements of disease that can be measured on a C.T. scan of the head and neck will be documented. The location and size must be recorded prior to treatment. A photograph of the area



will be taken at a fixed distance from the lesion. The area will be measured in 2 dimensions (product of longest and perpendicular dimensions).

6.6 A pretreatment blood sample will be collected and stored for analysis of leukocytes and serum.

6.7 Biopsy specimens, which have been previously obtained and have been confirmed for recurrent squamous cell of the head and neck, will be microdissected and analyzed for p53 mutations using SSCP analysis and direct DNA sequence analysis. The presence or absence of p53 mutations will not, however, be an exclusion criteria for eligibility.

## 7.0 EVALUATION DURING STUDY

7.1 Patients will have a CBC, platelet count, PT, PTT, SMA-12, electrolytes, and a chest x-ray prior to each course of therapy. Serum will be collected pre- and post-treatment for analysis of antibodies to adenovirus proteins.

7.2 History and physical with performance status and weight should be recorded before each course.

7.3 The tumors will be photographed clinically or endoscopically (depending upon site of disease) at the beginning of each course. Tumor measurements are to be recorded before each course for all measurable tumors.

7.4 All relevant information regarding viral dosage, tumor response, laboratory examinations, and treatment-related toxicities must be recorded before each treatment is given.

### 7.5 Parameters to be Measured In Vitro

7.5.1 Core biopsies or incisional biopsies will be obtained of accessible local and/or regional tumor. Tumor specimens will be collected 72 hrs. after the last injection of the adenovirus during the first treatment cycle. Tissue will be fixed immediately in 4% paraformaldehyde and 0.5% glutaraldehyde at 4°C and 10% formalin for histopathologic verification and evaluation. This will permit extraction of DNA and RNA and permit in situ hybridization.

7.5.2 Biopsies will be analyzed for incorporation of the transduced gene into the host genomic DNA and expression of the transduced gene at the RNA level by standard hybridization techniques following polymerase chain reaction. Pre- and post infected tissue biopsies will be evaluated histopathologically for any cytomorphologic changes. Tissue blocks will be retained for further evaluation of the p53 by immunohistochemistry and in-situ hybridization.

7.5.3 All patients will be evaluable for response and toxicity following one course of therapy.

7.6 An autopsy will be requested and immediately performed on all patients enrolled in the protocol who die. DNA will be extracted from tumor and normal tissues to determine if the adenovirus genes are present and expressed. PCR amplification of specific sequences for the adenovirus vector will be used to determine this. The following organ tissues will be analyzed in consented necropsies by PCR and routine light microscopy: upper aerodigestive tract mucosa, blood, brain, peripheral nerves (radial nerve), lung, liver, thyroid, adrenals, kidney, gastric, small bowel, and colonic mucosa, bladder mucosa, pancreas, gonad (testicles or ovaries, respectively), breast tissue, bone, pectoralis muscle, large and intermediate size vascular tissue (aorta and carotid), and skin.

7.7 A blood sample will be collected three times at one-half hour intervals following injection of the adenovirus. These samples will provide leukocytes to analyze for uptake of adenovirus DNA. Serum will be tested for antibodies to adenovirus proteins. This will be done by western blot analysis performed by Microbiological Associates, Inc. (Rockville, MD). Patients will be tested monthly during treatment, monthly for the first three months following completion of treatment, every three months for the remainder of the year following completion of treatment, and then at least yearly thereafter. Urine and sputum samples are analyzed daily following virus treatment for adenovirus antibodies.

- 7.8 Normal tissue samples will be collected during the follow-up visits and endoscopies. These will include samples of non-malignant mucosa, lymphocytes, and germ cells, if possible. These tissues will be analyzed for incorporation of the adenovirus.
- 7.9 A staging CT scan of the head and neck to evaluate local and regional disease will be obtained on an every three month basis during treatment.

#### 8.0 CRITERIA FOR RESPONSE AND TOXICITY

- 8.1 The graded toxicity scale used in this study has been previously described<sup>29</sup>. Three patients will be initially entered at each dose level in each group (resectable and non-resectable). If one patient in the cohort of 3 develops grade 3 toxicity for any system except hematologic (grade 4 required), an additional 3 patients will be entered at that dose level. If 2 of the 6 patients develop grade 3 (grade 4 hematologic) or greater toxicities, the next lower dose level not causing these toxicities is declared the Maximum Tolerated Dose (MTD). If MTD toxicity were to occur, patients could continue treatment at the next lower dose level. The MTD will be determined separately for each phase of the study. If MTD toxicity occurs in a cohort of 3 patients, then the next 3 patients may bypass the adenovirus alone phase and initially receive the adenovirus and cisplatin to establish the MTD for this combination.
- 8.2 The tumor bed and or neck will be photographed prior to each course of therapy for aerodigestive tract primary lesions.
- 8.3 The longest diameter and its perpendicular will be measured will be determined for measurable lesions. Size will be reported as the product of the diameters.
- 8.4 The rate of regrowth of the tumor will be calculated from the above measurements.
- 8.5 Patients will be evaluable for response if they have received at least one course of therapy. A complete response is defined as disappearance of all clinical evidence of tumor without the appearance of new lesions for a period of at least four weeks. Patients evaluable for a less-than-complete response must have had a bidimensionally measurable tumor. Partial response is defined as a 50% or greater reduction in the sum of the products of the diameters of the measurable disease; a minor response is defined as a 25% to less than 50% reduction in the sum of the products of the diameters of the measurable lesion. Patients are designated as having progressive disease if they show a 25% or greater increase in the size of their disease or if they develop unequivocal new lesions during treatment, and having no change if they have any tumor change not meeting the criteria described above.
- 8.6 The time to progression will be measured from the first observation with reduction in tumor bulk until there is evidence of progressive disease. Progressive disease is defined as an increase of  $\geq 25\%$  in the sum of the products of the diameters of the measured lesion. Patients must have received at least two courses of therapy before a designation of progression is made. The survival of patients will be measured from entry into protocol.
- 8.7 Alternative biologic endpoints will also be monitored. Pre-therapy and three days following the final treatment biopsies will be obtained and analyzed as described in section 7.5.2. Percentage of transfected cells in 3 random 100 x magnification fields will be determined. Maximal transduction rate will be determined by in-situ and immunohistochemically.
- 8.8 All toxicities encountered during the study will be evaluated according to the grading system (0-4) in Appendix C and recorded prior to each course of therapy. Duration of the toxicity and its treatment will be recorded. Life-threatening toxicities should be reported immediately to the Study Chairperson, who in turn, must notify the IRB, RAC, and FDA.

#### 9.0 CRITERIA FOR DISCONTINUING THERAPY

- 9.1 Progression of obstructing airway tumor that has recurred after a minimum of 2 courses of treatment.
- 9.2 The development of unacceptable toxicity defined as unpredictable, irreversible, or Grade 4 (non-hematologic). Patient refusal of therapy due to a specific toxicity should be graded as 4 and an explanatory note recorded.
- 9.3 Non-compliance by patient with protocol requirements.
- 9.4 Patient refusal to continue treatment.
- 9.5 Criteria for removal from protocol:
  - a) Refusal to continue study participation
  - b) Significant hemoptysis
  - c) Coagulopathy
  - d) Progressive pneumonitis or other infectious processes.

## 10.0 DATA AND PROTOCOL MANAGEMENT

- 10.1 Protocol Compliance: The attending physician and oncology research nurse must see patients prior to drug administration. All required interim and pretreatment data should be available and the physician must have a designation as to tumor response and toxicity grade.
- 10.2 Data Entry: Data must be entered into the Clinical Data Management System before a course of therapy can be given. A brief explanation for missing data should be recorded as a comment.
- 10.3 Accuracy of Data Collection: The Study Chairperson will be the final arbiter of response of toxicity should a difference of opinion exist.

## 11.0 STATISTICAL EVALUATION

Three patients will be entered at each dose level with 6 patients entered at the maximum tolerated or maximum attainable dose (limitations imposed by production of the adenovirus). A maximum of 21 patients will be entered into each study group, for a total of 42 patients for the entire study.

## 12.0 REFERENCES

1. Van der Riet, P., Nawroz, H., Hruban, R.H., Corio, R., Tokino, K., Koch, W., and Sidransky, D. Frequent loss of chromosome 9 p21-22 early in head and neck cancer progression. *Cancer Res.* 1994; **54**: 1156-1158.
2. Bishop JM. Molecular themes in oncogenesis. *Cell* 1991; **64**: 235-248.
3. Hollstein M, Sidransky D, Vogelstein B, Harris CC. *p53* mutations in human cancers. *Science* 1991; **253**: 49-53.
4. Lane DP, Benichou S. *p53*: oncogene or anti-oncogene? *Genes & Develop* 1990; **4**: 1-8.
5. Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR. Abnormal structure and expression of *p53* gene in human hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; **87**: 1973-1977.
6. Dolcetti R, Maestro R, Feriotto G, Pelucchi S, Rizzo S, Boiocchi M. *p53* genetic abnormalities in human squamous cell carcinomas of the larynx. *Oncogene* 1990; **6**: 44-45.
7. Rodrigues NR, Rowan A, Smith MEF, et al. *p53* mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990; **87**: 7555-7559.
8. Nigro JM, Baker SJ, Preisinger AC, et al. Mutations in the *p53* gene occur in diverse human tumor types. *Nature* 1989; **342**: 705-708.

9. Maestro, R., Dolcetti, R., Gasparotto, D., Doglioni, C., Pelucchi, S., Barzan, L., Grandi, E., and Boiocchi, M. High frequency of *p53* gene alterations associated with protein overexpression in human squamous cell carcinoma of the larynx. *Oncogene* 1992; 7: 1159-1166.
10. Chung, K.Y., Mukhopadhyay, T., Kim, J., Casson, A., Ro, J.Y., Goepfert, H., Hong, W.K., Roth, J.A. Discordant *p53* gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. *Cancer Res* 1993; 53: 1676-1683.
11. Mukhopadhyay T, Cavender A, Tainsky M, Roth JA. Expression of antisense K-ras message in a human lung cancer cell line with a spontaneous activated K-ras oncogene alters the transformed phenotype. *Proc Amer Assoc Cancer Res* 1990;31:304.
12. Soriano P, Montgomery C, Geske R, Bradley A. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* 1991;64:693-702.
13. Chen P-L, Chen Y, Bookstein R, Lee W-H. Genetic mechanisms of tumor suppression by the human *p53* gene. *Science* 1990;250:1576-1580.
14. Yonish-Rouach E, Resnitzky D, Rotem J, Sachs L, Kimchi A, Oren M. Wild-type *p53* induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature* 1991;352:345-347.
15. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J. Induction of apoptosis by wild-type *p53* in a human colon tumor-derived cell line. *Proc Natl Acad Sci USA* 1992;4495:4499.
16. Fujiwara T, Grimm EA, Mukhopadhyay T, Cai DW, Owen-Schaub LB, Roth JA. A retroviral wild-type *p53* expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res* 1993;53:4129-4133.
17. Lowe SW, Ruley HE, Jacks T, Housman DE. *p53*-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993;74:957-967.
18. Merino OR, Lindberg RD, Fletcher GH. An analysis of distant metastasis from squamous cell carcinoma of the upper respiratory and digestive tracts. *Cancer* 1977; 40: 147-156.
19. Votava C Jr, Fletcher GH, Jesse RH Jr, Lindberg RD. Management of cervical nodes, either fixed or bilateral, from squamous cell carcinoma of the oral cavity and faucial arch. *Radiology* 1972; 105: 417-420.
20. Komaki R, Garden AS, Cundiff JH. Endobronchial Radiotherapy. In: Roth JA, Hong WK, Cox JD, eds. *Advances in Lung Cancer*. Cambridge, MA: Blackwell Scientific Publications, Inc., 1992.
21. Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B, Schaffner W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 1985;41:521-530.
22. Mcgrory WJ, Bautista DS, Graham FL. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 1988;163:614-617.
23. Graham FL, Eb VD. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973;52:456-467.
24. Zhang WW, Fang X, Branch CD, Mazur W, French BA, Roth JA. Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. *BioTechniques* 1993;
25. Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ, ed. *Methods in Molecular Biology, Gene Transfer and Expression Protocols*. New Jersey: The Humana Press Inc, 1991:109-128.
26. Dai Y, Roman M, Naviaux RK, Verma IM. Gene therapy via primary myoblasts: long-term expression of factor IX

protein following transplantation in vivo. Proc Natl Acad Sci USA 1993;89:10892-10895.

27. Georges RN, Mukhopadhyay T, Zhang YJ, Yen N, Roth JA. Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct. Cancer Res 1993;53:1743-1746.
28. Casson AG, Mukhopadhyay T, Cleary KR, Ro JY, Levin B, Roth JA. p53 gene mutations in Barrett's epithelium and esophageal cancer. Cancer Res 1991;51:4495-4499.
29. Ajani JA, Welch SR, Raber MN, Fields WS, Krakoff IH. Comprehensive criteria for assessing therapy-induced toxicity. Cancer Invest 1990;8 (2):147-159.

Appendix A: Evaluation Before and During Therapy

Appendix B: Zubrod Scale of Performance Status

Appendix C: Toxicity Criteria

Appendix D: Figures/Figure Legends

Appendix E: Biosafety Procedures

Appendix F: Informed Consent

# APPENDIX A EVALUATION BEFORE AND DURING THERAPY

	Pre-Study	After completion of each course*	At two week intervals	Before each subsequent course	After completion of 1st course and every 3 mos
History	x	x	x	x	
PE	x	x	x	x	
Staging examination					
C.T. examination	x				x
Tumor size and response	x	x	x	x	
CBC, diff,	x	x	x***	x	
platelets, PT/PTT	x			x	
SMA-12	x	x	x***	x	
Serum Electrolytes	x	x	x	x	
Chest x-ray	x	x	x	x	
Toxicity and perf. status notation	x	x	x	x	
Completion of protocol specific template on Data Management System**	x	x	x	x	

\* Any studies necessary to completely evaluate malignant and concurrent non-malignant diseases and drug toxicity must be obtained and recorded at baseline and before each course as appropriate.

\*\* Any studies requested or added to protocol-specific data template of Clinical Data Management system must be collected as specified.

\*\*\* Weekly if indicated

Appendix B  
Performance Status Scales

Karnofsky Performance Scale (1)

Point	Description
100	Normal; no complaints; no evidence of disease
90	Able to perform normal activity; minor signs/symptoms of disease
80	Normal activity with effort; some signs/symptoms of disease
70	Cares for self; unable to perform normal activity or do active work
60	Requires occasional assistance; but is able to care for most of personal needs
50	Requires considerable assistance and frequent medical care
40	Disabled, requires special care and assistance
30	Severely disabled; hospitalization indicated; death not imminent
20	Very sick; hospitalization and active supportive treatment necessary
10	Moribund; fatal processes progressing rapidly
0	Dead

Zubrod Performance Scale (2)

Point	Description
0	Normal activity; asymptomatic
1	Symptomatic; fully ambulatory
2	Symptomatic; bed <50% of time
3	Symptomatic; in bed 50% of time; not bedridden
4	100% bedridden
5	Dead

THE UNIVERSITY OF TEXAS  
M. D. ANDERSON CANCER CENTER

Protocol Title: Clinical Protocol for Modification of Tumor Suppressor Gene  
Expression in Head and Neck Squamous Cell Carcinoma (HNSCC)  
with an Adenovirus Vector Expressing Wild-type p53

1. Participant's Name I.D. Number

You have the right to know about the procedures that are to be used in your participation in clinical research so as to afford you an opportunity to make the decision whether or not to undergo the procedure after knowing the risks and hazards involved. This disclosure is not meant to frighten or alarm you; it is simply an effort to make you better informed so that you may give or withhold your consent to participate in clinical research. This informed consent does not supersede other consents you may have signed in other protocols.

DESCRIPTION OF RESEARCH

2. PURPOSE OF THE STUDY: Some cancers that occur in the head and neck area may be due to a defect in a gene called p53. The purpose of this clinical research study will be to see whether a normal copy of the p53 gene can be placed inside the patient's cancer cells and cause the cancer to grow more slowly or to stop growing.
3. DESCRIPTION OF RESEARCH: To introduce the normal p53 gene into tumor cells utilizing a defective virus of the adenovirus type which is similar to other viruses that cause the common cold. This defective virus is called a "vector". These tumors will be injected directly with the adenovirus three times a week for two weeks. After two weeks of rest from receiving treatment, tumors will then be injected again with the virus three times a week for two consecutive weeks. If the tumor can be removed in its entirety, surgery will be performed for removal of tumor that is considered completely removable by the patient's physician. Surgery must be performed within four days after completion of the last injection of virus. At the time of surgery, additional virus is placed into the area where the tumor has been removed. Tubing, which is ordinarily placed in surgical areas, is used to allow for drainage of fluids from the surgery area. Three days after the tumor has been removed, virus will be placed into the tubing and allowed to enter the surgical site once again. If the patient has undergone surgery, this will be the last treatment with adenovirus.

If the cancer cannot be removed by the physician, this tumor will be injected directly



three times a week for two consecutive weeks. After two weeks of rest from treatment, courses of three injections weekly for two consecutive weeks are repeated on a monthly basis. Injections of adenovirus are continued if tumor continues to shrink. If there is absence of tumor shrinkage, evidence of tumor growth, or adverse reactions to the adenovirus injections, treatment will be terminated.

The injections into the tumor are delivered with a skinny needle. If the tumor is greater than approximately two inches in size, approximately two teaspoons of virus will be injected into the tumor. Smaller tumors, less than two inches, will be injected with less than one teaspoon of virus. Multiple injections of virus into the tumors will be required with these injections being spaced apart by approximately one-half inch each. Numbing medication may be placed on the overlying skin to decrease discomfort from injections.

Patients with tumors of the throat or the voice box may also receive the treatment. It may be necessary to remove a part of the tumor surgically or with a laser before the treatment with p53 is given. Other patients may require that a tracheostomy is first performed. A tracheostomy is a surgical operation to make an opening into the windpipe. Certain routine diagnostic studies will be performed before entry into this trial. These involve local examination of the tumor by inserting an instrument with a light into the throat.

If previous specimens are insufficient for laboratory studies related to this research, additional biopsies will be needed. The treatment will be repeated monthly as long as there is evidence that the tumor is not growing.

The experimental treatment and costs related to the patient's participation in this research and which include clinical examinations, biopsies, and other forms of testing will be provided free to the patient. A maximum of 42 patients will be entered in this study. Twenty one patients may have injections of adenovirus with surgery and twenty one patients may have injections of adenovirus without surgery.

The patient's course will be followed indefinitely. Dr. Clayman's office should be notified if an address change is made.

**PERMISSION FOR AUTOPSY:** In case of death, the family of the patient will be asked for permission to perform an autopsy.

#### 4. **RISKS, SIDE EFFECTS AND DISCOMFORTS TO PARTICIPANTS:**

Two small additional biopsies will be required in addition to the initial biopsy. Risks from biopsies include coughing up blood which is usually slight. Severe hemorrhage which requires emergency treatment is rare. Biopsy of neck masses may also be associated with a slight risk of bleeding or infection. This clinical research study may involve unforeseeable risks to the participant.

*Possibility of Causing a New Cancer.* It is possible that the research could cause cancer in normal cells although this risk is believed to be small when the injected virus has been properly safety tested prior to its use. The adenovirus vector has only been used, to date, on the lining of the breathing tube in over twenty patients with no ill effects noted.

- 4a. This clinical procedure may involve unforeseeable risks to the unborn children, therefore, the participants should practice adequate methods of birth control throughout the period of their involvement in the clinical study if they are sexually active. To help prevent injury to children, the female participants should refrain from breast feeding during participation in the clinical research study.

5. **POTENTIAL BENEFITS:**

This treatment may result in shrinkage of the tumor, which may decrease cancer associated symptoms or may prolong life.

6. **ALTERNATE PROCEDURES OR TREATMENTS:**

Chemotherapy or other experimental drugs may be an alternative for some individuals. These treatments cause shrinkage of cancer in a number of patients. Another option is to only control the symptoms of disease and not take chemotherapy treatment.

**UNDERSTANDING OF PARTICIPANTS**

7. I have been given an opportunity to ask any questions concerning the procedure involved and the investigator has been willing to reply to my inquiries. This procedure will be administered under the above numbered, title, and described clinical research protocol at this institution. I hereby authorize Dr. \_\_\_\_\_ the attending physician/investigator and designated associates, to administer this procedure.
8. I have been told and understand that my participation in this clinical research study is voluntary. I may decide not to participate, or withdraw my consent and discontinue my participation at any time. Such action will be without prejudice and there shall be no penalty or loss of benefits to which I may otherwise be entitled, and I will continue to receive treatment by my physician at this institution.

Should I decide not to participate or withdraw my consent from participation in this clinical research, I have been advised that I should discuss the consequences of my

effects of my decision with my physician.

In addition, I understand that the investigator may discontinue the clinical research study if, in the sole opinion and discretion of the investigator, the study or treatment offers me little or no future benefit, or the supply of medication ceases to be available or other causes prevent continuation of the clinical research study. The investigator will notify me should such circumstances arise and my physician will advise me about available treatments which may be of benefit at that time.

I will be informed of any new findings developed during the course of this clinical research study which might be relevant to my willingness to continue participation in the study.

9. I have been assured that confidentiality will be preserved except that qualified monitors from the Food and Drug Administration, Microbiological Associates, Magenta Corporation (manufacturers of the virus), and National Cancer Institute may review my records where appropriate and necessary. Qualified monitors shall include assignees authorized by the Surveillance Committee of this institution provided that confidentiality is assured and preserved. My name will not be revealed in any reports or publications resulting from this study, without my expressed consent.
10. I have been informed that, should I suffer any injury as a result of participation in this research activity, reasonable medical facilities are available for treatment at this institution. I understand, however, that I cannot expect to receive any credit or reimbursement for expenses from this institution or any financial compensation from this institution for such injury.
11. I have been informed that I should inquire of the attending physician whether or not there are any services, investigational agents or devices, and/or medications being offered by the sponsor of this clinical research project at a reduced cost or without cost.

Costs related to my medical care will be covered by the Introgen Sponsored Research Agreement. Clarification of specific cost issues may be addressed in section 3 of this informed consent. I have been given the opportunity to discuss the expenses or costs associated with my participation in this research activity.

12. It is possible that this research project will result in the development of beneficial treatments, devices, new drugs, or possible patentable procedures, in which event I understand that I cannot expect to receive any compensation or benefits from the subsequent use of information acquired and developed through my participation in this research project.
13. I understand that refraining from breast feeding and practicing effective contraception is medically necessary and a prerequisite for my participation in this clinical research

study. Should contraception be interrupted or if there is any suspicion of pregnancy, my participation in this clinical research study will be terminated at the sole discretion of the investigator.

14. I may discuss any questions or problems during or after this study with Dr. Gary L. Clayman at \_\_\_\_\_ In addition, I may discuss any problems I may have or any questions regarding my rights during or after this study with the Chairman or the Surveillance Committee at \_\_\_\_\_ and may in the event any problem arises during this clinical research contact the parties named above.

### CONSENT

Based upon the above, I consent to participate in the research and have received a copy of the consent form.

\_\_\_\_\_  
DATE

\_\_\_\_\_  
SIGNATURE OF PARTICIPANT

\_\_\_\_\_  
WITNESS OTHER THAN PHYSICIAN  
OR INVESTIGATOR

\_\_\_\_\_  
SIGNATURE OF PERSON  
RESPONSIBLE & RELATIONSHIP

I have discussed this clinical research study with the Participant and/or his or her authorized representative using a language which is understandable and appropriate. I believe that I have fully informed this participant of the nature of this study and its possible benefits and risks, and I believe the participant understood this explanation.

\_\_\_\_\_  
PHYSICIAN/INVESTIGATOR

Appendix C  
Toxicity Criteria

	Grade 1	Grade 2	Grade 3	Grade 4
ALLERGY				
ACUTE ALLERGIC REACTION	Transient rash, Drug fever <38C/100.4F	Urticaria, Drug fever >38C/100.4F mild bronchospasm	Serum sickness, bronchospasm, req parenteral meds	Anaphylaxis
FEVER WITH DRUG	<38C, <100.4F	38-40c, 100.4-104F	>40C/>104F despite antipyretic rx	Fever & hypertension
CARDIOVASCULAR.				
ARRHYTHMIA	Resting sinus tech, PAC's, <1 PVC/hr. nonspec S-T or wave abn; 1st (dog) AV block	Sustained atrial arrhythmia, 1-9 PVC, hrs. ischium ST or T wave, Mobitz type I, incompl or rate-related bundle branch block	10-20 PVC/hr. multifocal PVC couplets, 3-5 consec PVC and salvos, ing pattern on EKG, Mobitz type II, Bundle branch or bifascic block	>30 PVC/hr. 6 consec PVC MI, 3rd dec AV block
CARDIAC SYMPTOMS	Mild or transient	Symptoms on exertion	Symptoms at rest, persistent	Severe symptoms, non-response to rx
CARDIAC BIOPSY	0.5	1.0	1.5	>1.5
EJECTION FRACTION ABNORMALITY	>60-64%	50-59%	40-49%	<40%
HYPERTENSION	10-20% dec systol	21-30% dec systol	31-40% dec systol, req pressors	>40% dec systol, not response to press
PERICARDIAL EFFUSION	Small	Moderate	Large, no tamponade	Large, tamponade
PERIPH CAPILLARY LEAKAGE	Min ankle pitting edema	Ankle pitting edema & wt. gain <10 lbs.	Periph edema, wt. gain >9.9 lbs, pleural effusion w/no pul fx deficit	Anasarca, sev pleural effusion w/pul rx deficit, asc

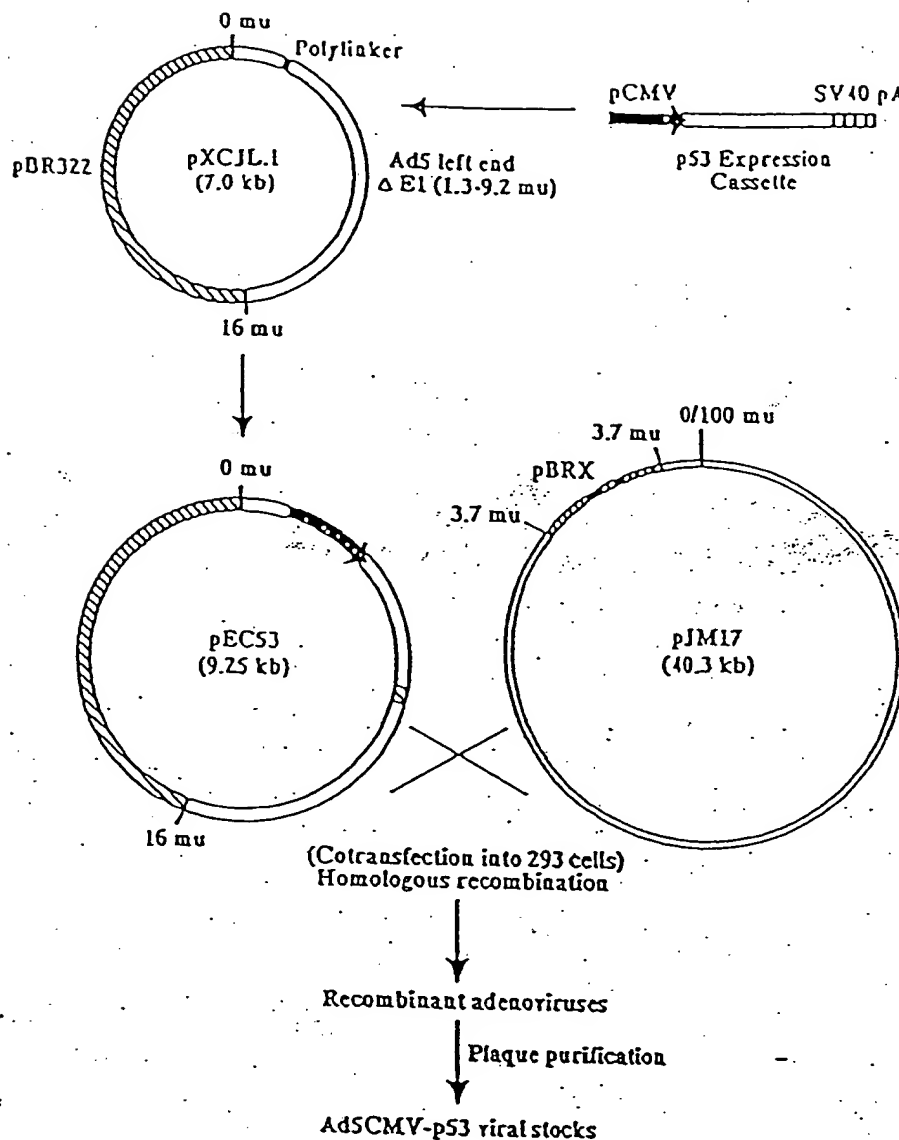
	Grade 1	Grade 2	Grade 3	Grade 4
CNS				
AFFECT ABNORMALITY	Transient panic/apathy	Sustained panic/apathy	Sustained panic/apathy, rx req	Sustained panic/apathy nonresp to rx
ATAXIA	Mild/transient gait or limb	Mod gait or limb ataxia	Mod gait & limb ataxia	Disabling ataxia, b
AUTONOMIC DYSFUNCTION	Abnormal sweating	Impotence	Asympt arrhythmia, orthostat hypotension	Sympt arrhythmia, orthostat hypotensi.
BLADDER DYSFUNCTION AUTONOMIC	-----	Dysfunction not req cath	Req cath	Req permanent cath
COGNITIVE DEFICIT	Slow, accurate	Impaired memory/new learning	Global deficiency	Unresponsive
CONSTIPATION AUTONOMIC	Mild, no rx req	Occasionally req cathartics	Daily cathartics/enema req	Abdominal distention, vomitin
LEVEL OF CONSCIOUSNESS	Drowsy, easily arousable	Response to loud or tactile stimuli	Response to pain only	Unresponsive to any stimuli
FOCAL SEIZURES	Isolated	<3/day	>2/day	Status epilepticus
GENERALIZED SEIZURES	Isolated	<3/day	>2/day	Epilepsia partialis continue
HEARING LOSS	Transient decrease, tinnitus	Moderate loss	Severe loss	Deaf
LANGUAGE ABNORMALITY	Word finding difficulty	-----	Reduced verbal output or comprehension	Global aphasia
MOTOR DEFICIT	Mild/transient weakness	Mod weakness, ambulatory	Non-ambulatory	Complete paralysis

	Grade 1	Grade 2	Grade 3	Grade 4
MOVEMENT DISORDERS	Transient and limb movement	Mod limb/gait disorder	Severe and reversible parkinsonism, dystonia or tremor	Permanent parkinsonism tremor or dystonia
PERCEPTION ABNORMALITY	Occasional misperception	Brief unformed hallucination	Freq formed hallucination	Constant hallucinat
SENSORY DEFICIT	Paresthesias, decr DTSS	Mild objective abnormality, absent DTRs	Severe paresthesia, mod objective abnormality	Complete sensation
SPEECH ABNORMALITY	Mildly slurred	Moderate slurring	Unintelligible	Mute
VERTIGO	Mild, transient	Moderate, nausea	Associated w/N&V	Disabling, intractabl
VISION	Slight reduced acuity	Finger counting only	Light perception only	Blind
DERMATOLOGIC				
ALOPECIA	Partial loss	Complete loss	Non-reversible loss	
CHELITIS	Chapping	Fissures	Bleeding	Necrosis
SKIN REACTION	Local erythema, dry skin, mild or transient rash	Diffuse erythema, dry desquamation, macropap rash, pruritus	Moist desquamation, ulceration, bullous disease	Exfoliative dermatitis, necrosis, req surg
GASTROINTES-TINAL				
DIARRHEA	Transient, <2 days	Tolerable, >2 days	Intolerable, req/s rx	Hemorrhagic, dehydrat
NAUSEA AND VOMITING	Mild nausea alone	Mod nausea, transient vomiting	Severe nausea or vomit, req's rx	Intract vomiting req hosp.

	Grade 1	Grade 2	Grade 3	Grade 4
STOMATITIS	Soreness, erythema	Erythema, patchy, ulceration can eat solids	Confluent ulceration, liquid diet	Hemorrhagic ulceration, necrosis, req parenteral support
GENERAL				
ACHING PAIN	Mild	Moderate	Severe	Intractable
CHILLS	Mild	Moderate	- - - -	- - - -
HEMATOLOGIC				
ANEMIA	9.5-10.9	8.0-9.4	6.5-7.9	<6.5
GRANULO-CYTOPENIA	1.5-1.9	1.0-1.4	0.5-0.9	<0.5
HEMO-RRHAGE	Pepechise, min blood loss, no trans req	Blood loss req 1-2 U trans	Blood loss req 3-4 U trans	Blood loss req <4 U trans
LEUKOPENIA	3.0-3.9	2.0-2.9	1.0-1.9	<1.0
THROMBO-CYTOPENIA	75-99	50-74	25-49	<25
HEPATIC				
ALK PHOS	1.5-2.5 x N, MDACC 165-275	2.6-5 x N, MDACC 276-550	5.1-20 x N, MDACC 551-1100	>10 x N, MDACC >1100
BILIRUBIN INCREASE	1.5-2.5 x N, MDACC 1.5-2.5	2.6-5 x N, MDACC 2.6-5	5.1-20 x N, MDACC 5.1-10	>10 x N, MDACC >10
HEPATIC SYMPTOMS	- - - - -	- - - - -	Precoma	Hepatic coma
TRANSAMINASE INCREASE	1.5-2.5 x N, MDACC SGOT 45-75, SGPT (SMA) 60-100	2.6-5 x N, MDACC SGOT 76-150, SGPT (SMA) 101-200	5.1-20 x N, MDACC SGOT 151-300, SGPT (SMA) 201-400	>10 x N, MDACC SGOT >300, SGPT (SMA) >400
INFECTION				
INFECTION	FUO	Minor infection	Major organ infection	Disseminated infection
PULMONARY				
FVC FUNCTION ABNORMALITY	FVC 70-80% pred, FEV1 or DLCO 60-80% pred, 15-25% dec from abn baseline	FVC 50-69% pred, FEV1 or DLCO 40-59% pred, 26-50% dec from abn baseline	FVC <50% pred, FEV1 or DLCO >40% pred, >50% dec from abn baseline	Unable to perform test due to resp dis
RESPIRATORY SYMPTOMS	Mild or transient	Symptoms on exertion	Symptoms at rest, persistent	Severe symptoms, nonresponse to rx
RENAL				
CREATININE	<1.25 x baseline	1.25-2.5 x baseline	2.6-5 x baseline	>5 x baseline
DYSURIA	Mild	Moderate	Severe	Unacceptable
HEMATURIA	6-10 RBC/HPF	11-50 RBC/HPF	Gross, >50 RBC/HPF	Clots, obstructive
PROTEINURIA	1+, <0.3g%, <3g/L	2-3+m 0, 3-1, 0g%, 2-10g/L	4+, >1.0g%, >10g/L	Nephrotic syndrome



## Appendix D



**Figure 1.** Scheme for generation of recombinant p53 adenovirus. The p53 expression cassette was inserted between the Xba I and Cla I sites of pXCJL.1. The p53 expression vector (pEC53) and the recombinant plasmid pJM17 were cotransfected into 293 cells. The transfected cells were maintained in medium until the onset of the cytopathic effect. Identification of newly generated p53 recombinant adenoviruses (Ad5CMV-p53) by PCR analysis of the DNA samples prepared from the cell culture supernatants was described elsewhere<sup>24</sup>.

## Appendix D

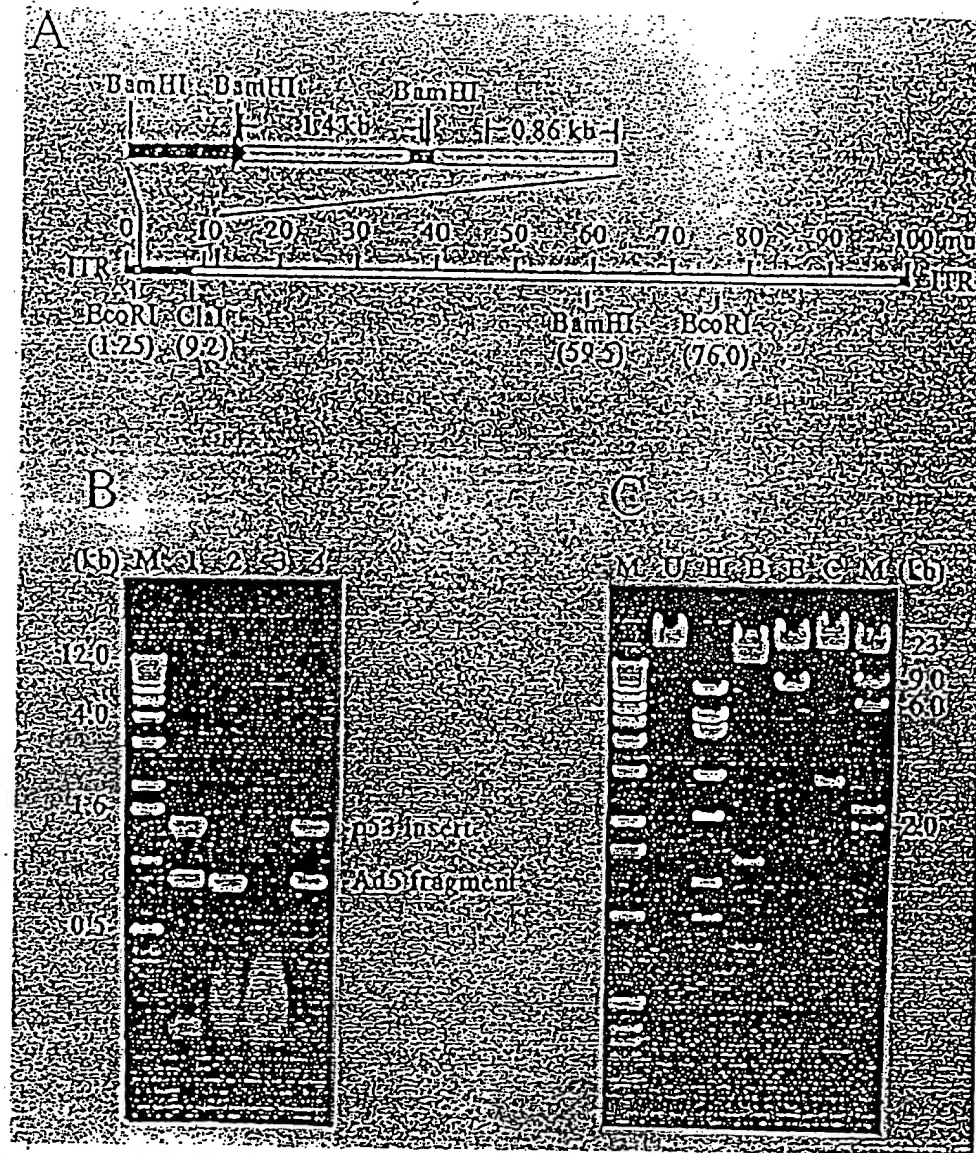
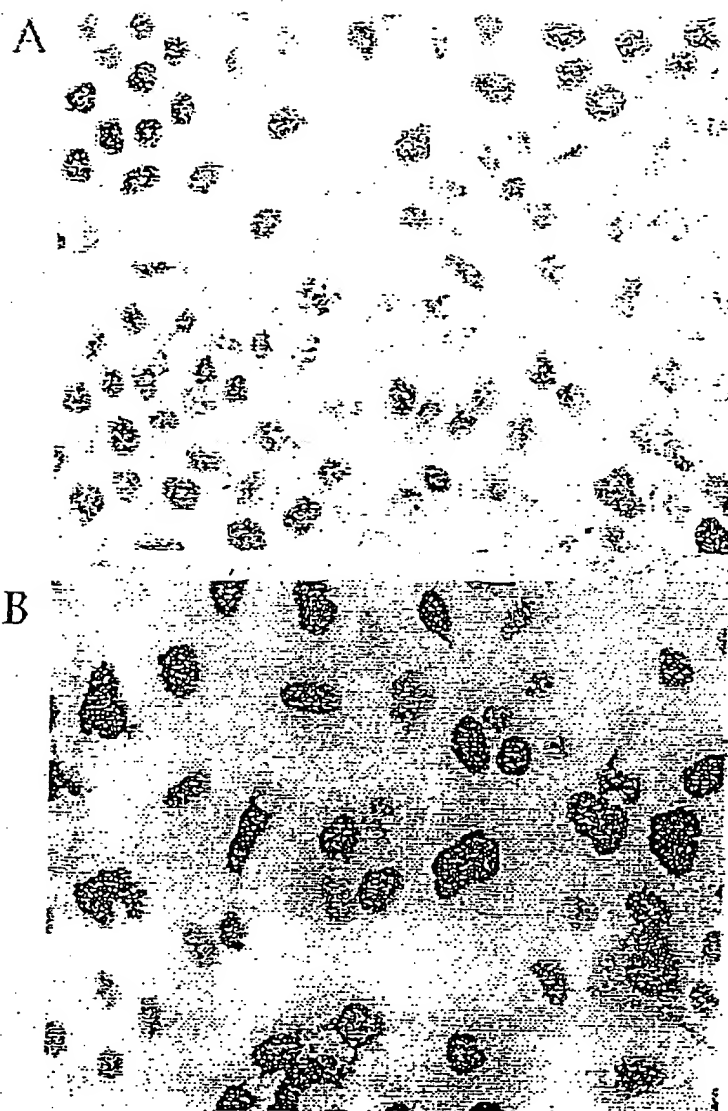


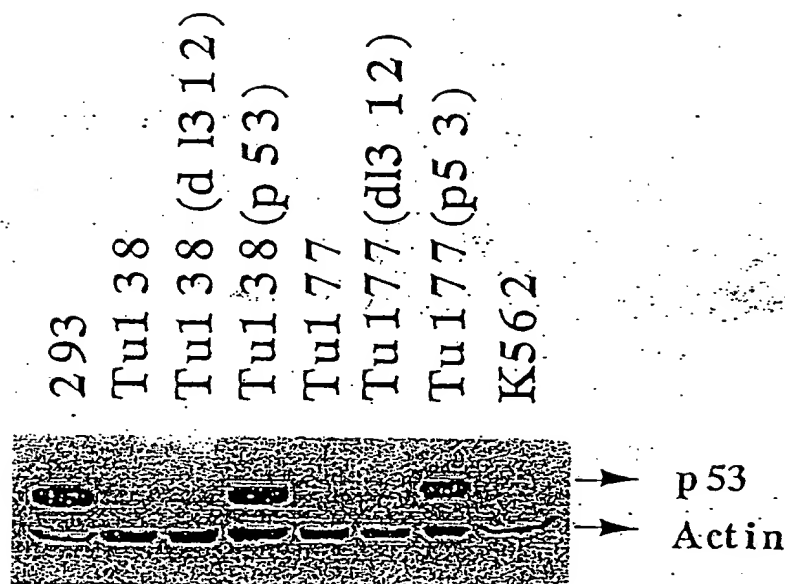
Figure 2. Structural analysis of Ad5CMV-p53 DNA. (A) A map of Ad5CMV-p53 genomic DNA, showing the locations of the p53 expression cassette, the PCR primers, and the restriction sites. (B) An agarose gel analysis of PCR products. Two pairs of primers that define 1.4-kb (p53) and 0.86-kb (Ad5) DNA fragments were used in each reaction. DNA templates used in each reaction were pEC53 plasmid (lane 1), Ad5/RSV/GL2 DNA (lane 2), no DNA (lane 3), and Ad5CMV-p53 DNA (lane 4). (C) Restriction mapping of Ad5CMV-p53 DNA. CsCl-gradient purified Ad5CMV-p53 DNA samples were digested with no enzyme (U), Hind III (H), Bam HI (B), Eco RI (E), and Cla I (C), respectively, and analyzed on 1% agarose gel.

## Appendix D



**Figure 3.** Infectivity of Ad5CMV-p53 in Tu138 cells. Tu138 cells were infected with Ad5CMV-p53 in dose response experiments or Ad5/RSV/GL2 at 50 PFU/cell for 24 h. Medium alone was used as a mock infection. The infected cells were analyzed by immunostainings. No differences in staining were noted comparing mock infection or Ad5/RSV/GL2. Absence of non-specific staining was confirmed by preabsorption methods. (A) Mock infection probed with anti-p53 antibody. (B) Ad5CMV-p53 infection probed anti-p53 antibody. The anti-p53 antibody used was PAb 1801, with the avidin-biotin method for staining.

## Appendix D



**Figure 4.** Relative level of expression of exogenous p53 in Tu138 and Tu177 cells. Samples of cells that were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 50 PFU/cell were prepared 24h after infection. The relative levels of expression of p53 were analyzed by Western blotting using anti-p53 and anti-actin antibodies. (A) Coomassie blue staining of an SDS-PAGE analysis, showing relative quantities of protein samples loaded. Western blot analysis 24-h post infection. Cell extracts isolated from cells were subjected to SDS-PAGE, blotted onto Hybond-ECL membranes, and probe with both anti-p53 and anti-actin antibodies. Lanes 1 and 8 are 293 and K562 cells, respectively. Lanes 2 and 5 are mock-infected Tu-138 and Tu-177 cells. Lanes 3 and 6 are Tu-138 and Tu-177 cells infected with replication-deficient adenovirus. Lanes 4 and 7 are Tu-138 and Tu-177 cells infected with Ad5CMV-p53.

## Appendix D

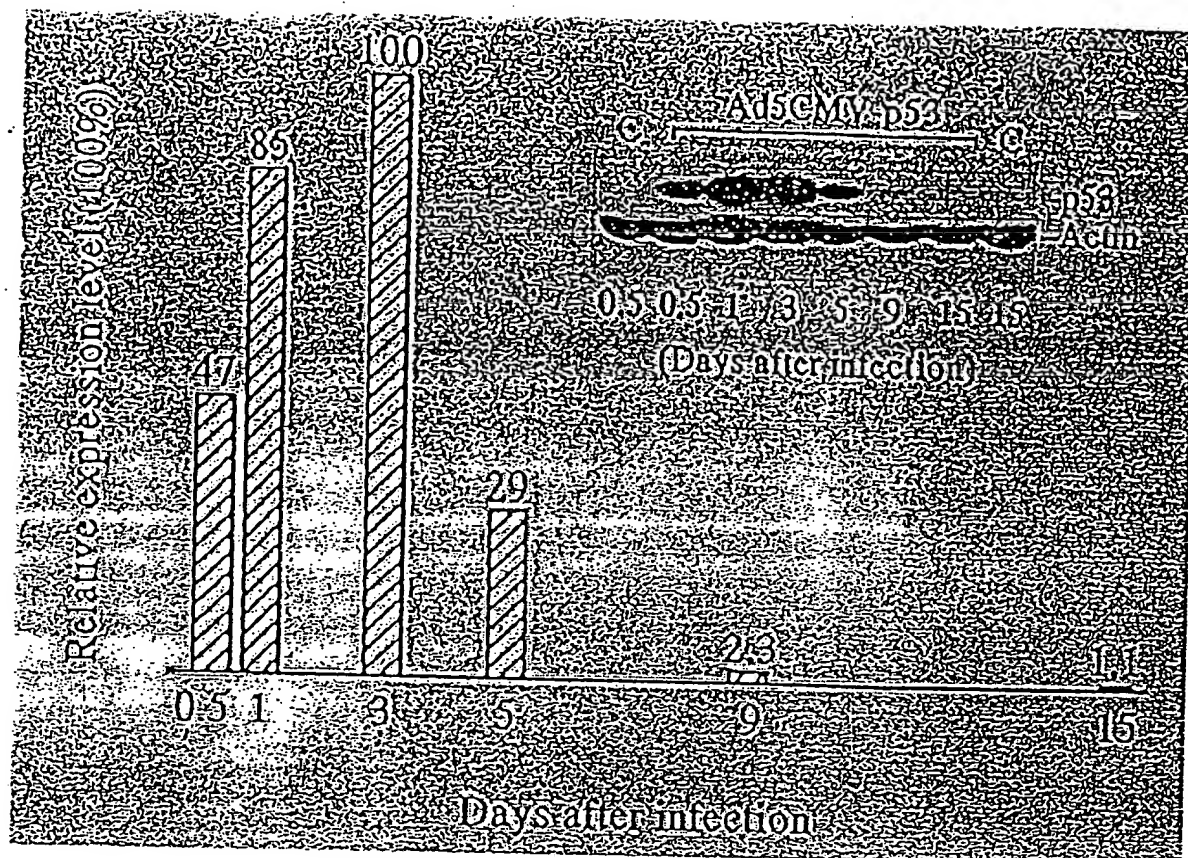


Figure 5. Time course of exogenous p53 expression in H358 cells. Multiple dishes of H358 cells were infected with Ad5CMV-p53 at 10 PFU/cell. Cell lysates were prepared at indicated time points after infection. Western blotting was probed with anti-p53 and anti-actin antibodies. Relative quantities of exogenous p53 were determined by densitometer.

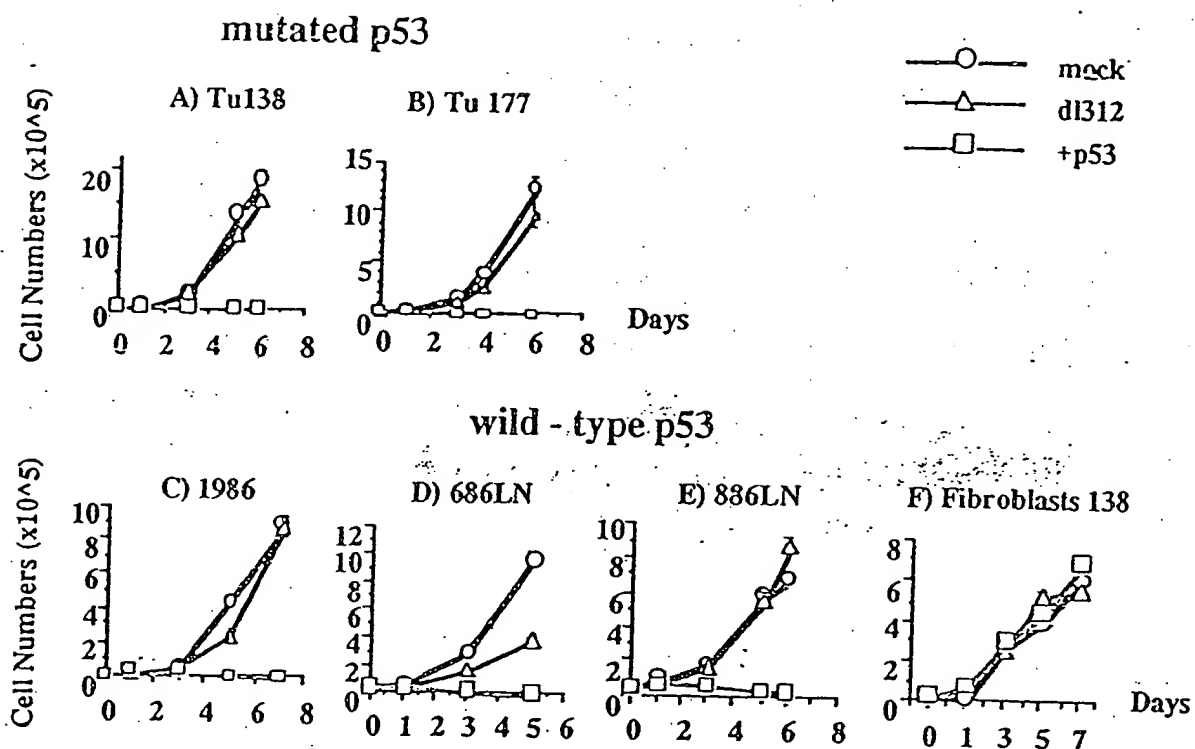


Figure 6. Growth curves of the Ad5CMV-p53-infected human head and neck squamous carcinoma and fibroblasts cells. The cells were inoculated at densities of  $1 \times 10^5$  in 60 mm culture dishes 24 h before infection. The cells were infected with Ad5CMV-p53 or Ad5/RSV/GL2 at 50 PFU/cell. Culture medium alone was used as mock infection. Triplets of each cell line for each treatment were counted daily from postinfection day 1 to day 6. The curves are plotted from the combined data of three experiments.

A) Tu138 B) Tu177 C) 1986 D) 686LN E) 886

F) Fibroblasts derived from Tu138 patient. Graphs A through E showed no viable tumor cell lines following 72 hours whereas non-malignant fibroblasts reveal normal growth (and morphology) characteristics.

## Appendix D

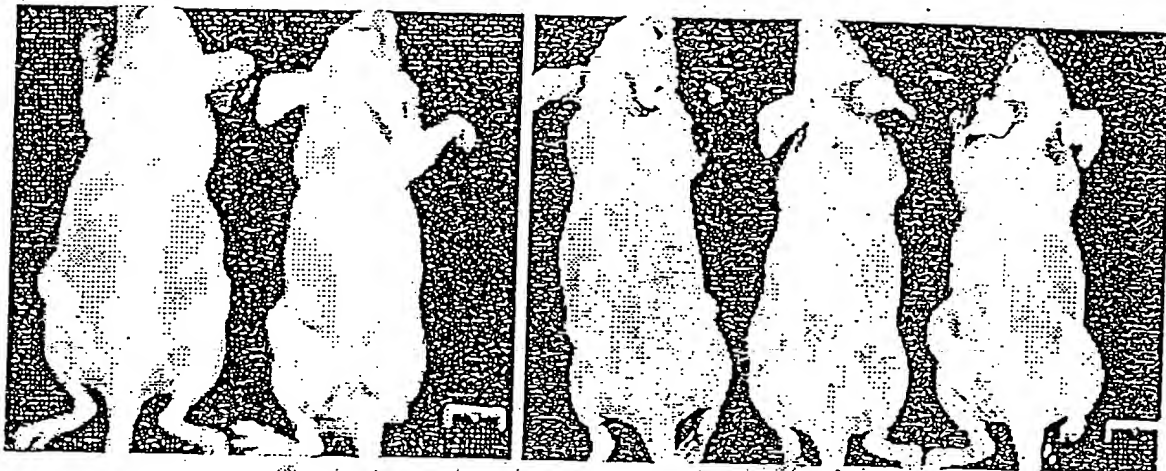


Figure 7. Inhibition of SCCN cell growth *in vivo*. Pictures of the representative nude mice studies for both Tu1-38 (left) and Tu-177 (right) cell lines. The effect of Ad5CMV-p53 on established subcutaneous tumor nodules was determined in nude mice in a defined pathogen free environment. Experiments were reviewed and approved by institutional committees for both animal care and utilization and for recombinant DNA research. Briefly, following induction of acepromazine/ketamine anesthesia, three separate subcutaneous flaps were elevated on each animal and  $5 \times 10^6$  cells in  $150 \mu\text{l}$  of complete media were injected subcutaneously into each flap using a blunt needle; the cells were kept in the pocket with a horizontal mattress suture. Seven animals were used for each cell line. After 4 days, the animals were re-anesthetized and the flaps were re-elevated for the delivery of  $100 \mu\text{l}$  of 1) Ad5CMV-p53 ( $10^8$  PFU) in the right anterior flap; 2) replication-defective virus ( $10^8$  PFU) in the right posterior flap; and 3) transport medium alone, in the left posterior flank. All injection sites had developed subcutaneous visual and palpable nodules before treatment was administered. Animals were observed daily and sacrificed on day 20.

## Appendix D

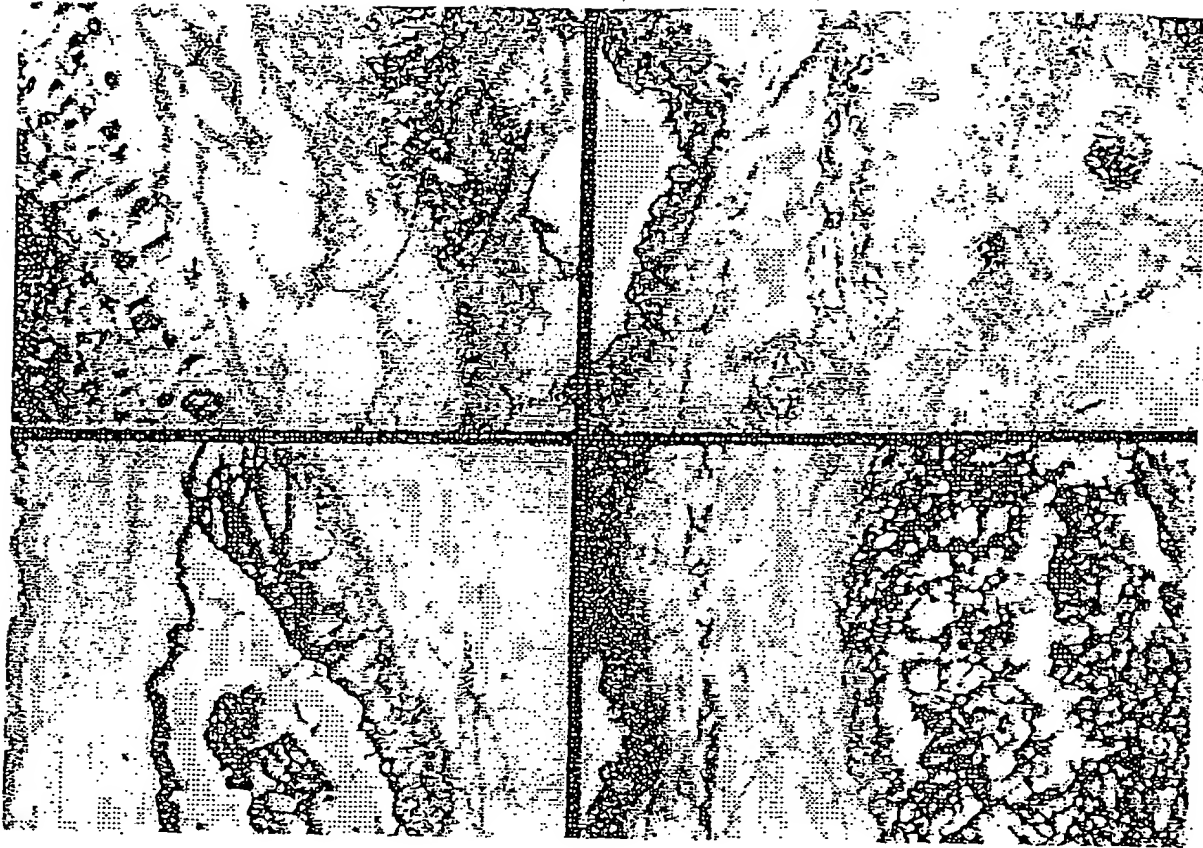
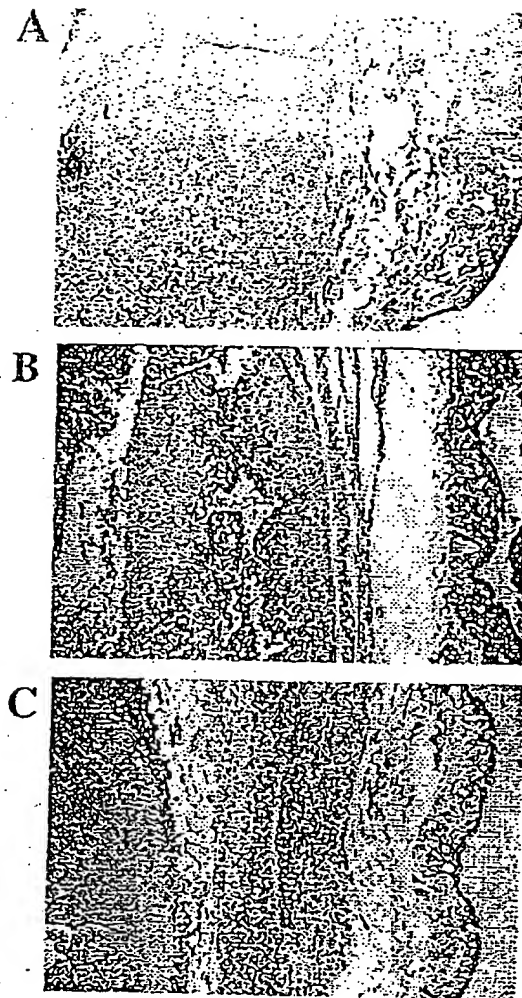


Figure 8. Dose response in-vivo infecting experiments in microscopic residual disease flap model. A marker virus AdCMVB-galactosidase exhibits blue X-gal staining within infected cells. Top left panel - Mock infection, Top right panel -  $10^7$  PFU/cell, Bottom left panel -  $10^8$  PFU/cell, Bottom right panel -  $10^9$  PFU/cell. A clear dose-response is noted. Inflammation and edema increase histologically with increasing viral titers. Magnification 63 X.





**Figure 9.** In-vivo infectivity of AdCMVp53 in microscopic residual disease flap model. The AdCMVp53 was pipetted into the subcutaneous flap 48h following tumor cell line delivery. A representative experiment of the wild-type p53 HNSCC cell line MDA686LN is shown. Panel A - mock infection showing lack of immunostaining in the wild-type p53 cell line, Panel B -  $10^8$  PFU/cell, Panel C -  $10^9$  PFU/cell. Immunostaining was performed using the polyclonal rabbit anti-human antibody OM1 (Signet Laboratories, Denham, MA) and the Avidin-biotin method. Basal immunostaining with viable tumor is seen in Panel A. Panel B shows peripheral tumor necrosis with immunostaining of in the more central portion of the tumor. Panel C reveals total necrosis of the tumor with immunostaining found in the entire surgical pocket with multiple layers expressing protein including stroma and superficial muscular layers. Magnification 100X.

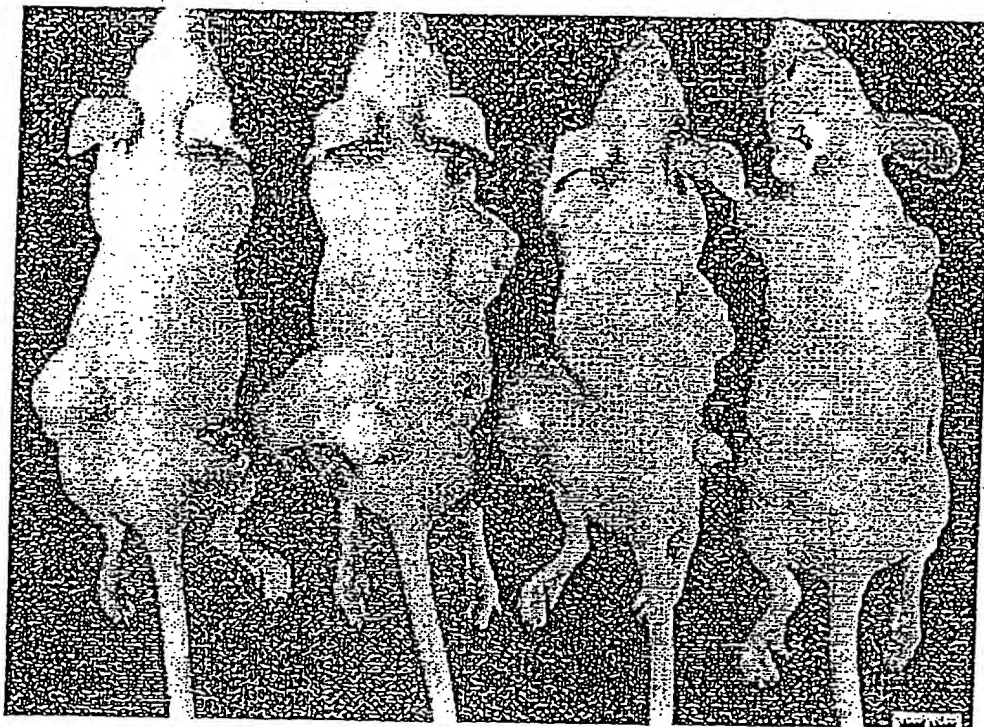


Figure 10.

Repeat in-vivo infection with AdCMVp53 on established tumors in nu/nu female mice. Established subcutaneous Tu138 tumors were allowed to develop to no greater than 1.5cm in greatest dimension in both right and left posterior flanks. Prior to the institution of adenovirus treatment there were no significant differences in tumor sizes in the posterior flanks. The left flank was peritumorally infiltrated with  $10^9$  PFU of dl 312 in 0.1 ml and the right flank with AdCMVp53 at the same dosage ( $10^9$  PFU). The animals were sacrificed due to tumor burdens and the above photograph taken. One animal was sacrificed earlier due to excessive tumor burden in the dl 312 site. A significant reduction in tumor mass is noted in the right flank. With reduction in tumor mass in the p53 treated sites, all animals developed skin loss and eschar formation. All animal experiments and care were performed in accordance with the University of Texas M.D. Anderson Cancer Center Animal Care and Use Committee.



## DEPARTMENT OF HEALTH &amp; HUMAN SERVICES

Public Health Service

Food and Drug Administration  
1401 Rockville Pike  
Rockville MD 20852-1448

Our Reference: BB-IND 6165

AUG 24 1995

The University of Texas M.D. Anderson Cancer Center  
Attention: Leonard Zwelling, M.D.  
Associate Vice President, Clinical & Translational Research

Dear Dr. Zwelling:

We have reviewed your Investigational New Drug Application (IND) for "Adenovirus Vector (Ad5CMV-p53; M.D. Anderson). Expressing Wildtype p53 Gene; Administered Intralesional or via Tumor Bed," and as relayed to you in our letter of your study may proceed; however, we have the following comments and requests for additional information:

## CLINICAL INFORMATION

1. Although the target population would appear to be patients who have failed conventional therapy, the eligibility criteria could be interpreted more broadly. It seems that patients who have residual disease following neoadjuvant irradiation or combined modality therapy are eligible for this protocol. Although these patients are not likely to have a durable complete response to conventional treatment, the informed consent should state more clearly that this is an experimental therapy, and that conventional surgical treatment is a reasonable alternative. Please submit the revised informed consent.
2. The protocol does not provide a clear definition of a treatment course. If injections administered three times per week for two weeks constitute a single course of treatment, this is stated clearly only in the informed consent. It is not clear if patients with multiple sites of disease will receive a fixed total dose of Ad5CMV-p53 equally divided between each injected site or if the total dose will vary according to the number of sites injected. If the latter is the case, then systemic exposure to the vector would increase with the number of sites. Please clarify the total and/or maximum dose of vector for each cohort.

Page 2 - BB-IND 6165

3. A basic assumption of the protocol is that there will be no treatment related toxicity. We note that only grade 4 toxicity prompts changes in the treatment plan. We strongly recommend that the administration of the vector be discontinued or decreased in the event of a grade 3 or greater toxicity or any toxicity which does not resolve to less than or equal to grade 1 toxicity by the next treatment course.
4. Dose escalation in cohorts above  $1 \times 10^9$  pfu should proceed at half-log increments rather than one log as currently specified in the protocol since doses at or above  $1 \times 10^9$  pfu are associated with significant local toxicity. A clinical trial in patients with non-small cell lung cancer of very similar design is also being conducted at your institution. If you have any information regarding the toxicity in the ongoing clinical trial in patients with lung cancer at doses above  $1 \times 10^9$  pfu which you feel may support a more aggressive dose escalation schema, please submit these data. In lieu of such data, we request that you submit a revised protocol with the modified dose escalation schema and plan for dose modification.

If you have any questions, please contact Jeanne Delasko at

Sincerely yours,

*Sharon T. Risso*

Sharon T. Risso  
Director  
Division of Application Review and Policy  
Office of Therapeutics  
Research and Review  
Center for Biologics  
Evaluation and Research

Revisions

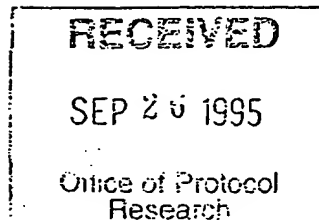
approved  
A658  
10-2-95

Aman U Buzdar MD

## INTEROFFICE MEMORANDUM

TO: Aman U. Buzdar, M.D.  
Chairman, Surveillance Committee

FROM: Gary L. Clayman, D.D.S., M.D. *PLC*  
Department of Head and Neck Surgery



DATE: September 25, 1995

SUBJECT: Administrative Approval of Revised Protocol HNS 94-001 entitled,  
"Clinical Protocol for Modification of Tumor Suppressor Gene  
Expression and Induction of Apoptosis in Head and Neck  
Squamous Cell Carcinoma (HNSCC) with an Adenovirus  
Vector Expressing Wildtype p53"

The FDA has mandated that an additional change be added to Protocol HNS 94-001.  
This change includes clarification of dose escalation in cohorts above  $1 \times 10^9$  pfu.

### NOTE:

#### Treatment Plan:

- 5.4 Dose Escalation: The adenovirus dose will increase in one  $\log_{10}$  increments for each group until  $10^9$  PFUs are needed. Thereafter, adenovirus doses will increase at half-log increments. Patients entered at a given dose level will not be eligible for dose escalation. We estimate based on the achievable titers of adenovirus that six dose levels can be done thus requiring 21 patients in each group (resectable and non-resectable).

This modification is included in the revised protocol. I am requesting administrative approval for this change. The revised protocol must be submitted to the FDA within 21 days.

GLC:lkM

Attachment

✓ cc: Leonard Zwelling, M.D.  
Michael J. Keating, M.D.

## PROTOCOL ABSTRACT

Protocol: (Give number and abbreviated title)

(Two lines not to exceed 75 characters per line)

Clinical Protocol for Modification of Tumor Suppressor Gene Expression and Induction of Apoptosis in Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus Vector Expressing Wildtype p53

Study Chairman:

Gary L. Clayman, D.D.S., M.D., Department of Head and Neck Surgery

Patient Eligibility:

(Twenty lines not to exceed 75 characters per line)

1. Patients must have histologic proof of squamous cell carcinoma of the head and neck. Patients must be either unable to receive conventional treatment (e.g. the patient received radiation therapy with or without surgery) or have failed conventional treatment. Those patients with extensive local or regional disease that have persisted or recurred following radiation therapy (with or without chemotherapy or surgery) and have clinically resectable, but likely non-curable (<10% disease free survival) are also eligible. Patients need not have received a trial of chemotherapy prior to entering this protocol. All eligible patients will be discussed at the Head and Neck Surgery Multidisciplinary Treatment Planning Conference prior to protocol enlistment.
2. Patients must have clinical evidence of advanced local and/or regional cancer which is unresectable or for which no meaningful resection with surgical margins will be obtainable.
3. All patients must have a life expectancy of at least 12 weeks and must have a performance status of  $\leq 2$  (Zubrod scale, Appendix B).  
All patients must sign an informed consent indicating that they are aware of the investigational nature of this study in keeping with the policies of the hospital. The only acceptable form is the one attached at the end of this protocol.
5. Patients will be tested for HIV prior to entry onto the protocol and must be HIV-negative. Patients with upper respiratory infections will not be treated until the infection resolves.
6. Patients must have adequate bone marrow function (defined as peripheral absolute granulocyte count of  $>2,000/\text{mm}^3$  and platelet count of  $100,000/\text{mm}^3$ ), adequate liver function (bilirubin  $\leq 1.5 \text{ mg/dl}$ ), and adequate renal function (creatinine  $<1.5 \text{ mg/dl}$ ).

Treatment Plan: (Include dose adjustment)

(Twenty lines not to exceed 75 characters per line)

1. The study will be an open-label upward dose ranging study for adenovirus-p53 vector (Ad5CMV-p53) in two patient groups. The two groups of patients will consist of a) resectable and b) non-resectable recurrent disease. The first phase of the study will allow assessment of toxicities related only to the vector. Patients will receive on intratumor injection of Ad5CMV-p53. The initial dose will be  $10^6$  plaque forming units (PFU).
2. Dose Escalation: The adenovirus dose will increase in one  $\log_{10}$  increments for each group until  $10^9$  PFUs are reached. Patients entered at a given dose level will not be eligible for dose escalation. We estimate based on the achievable titers of adenovirus that six dose levels can be done thus requiring 21 patients in each group (resectable and non-resectable).
3. All patients shall be registered with the Data Management Office
4. Patients with local-regional tumors will have injection of a total dose of 10 ml for tumors  $\geq 4 \text{ cm}$  in diameter or 3 ml for tumor  $<4 \text{ cm}$  in diameter of the adenovirus preparation with the appropriate number of viral particles at multiple sites percutaneously or transorally. The treatment will be repeated three times weekly for two weeks. Dose escalation may proceed after a minimum two week follow-up of the last patient entered into the previous dose level. Treatment will continue on a monthly basis as long as there is no tumor progression. After one year the patients will be evaluated for continuation of therapy.
5. Those patients with surgically resectable disease will be treated by tumoral injection of adenovirus preparation as described above. The treatment will be repeated for two consecutive courses. Within four days of completion of the second course, the patients will be eligible to proceed with surgical resection. Prior to surgical closure, 10 ml of adenovirus preparation will be administered into the surgical defect (operative bed) and allowed to remain in contact for 60 minutes. The wounds are then closed and drains placed.

- 5.3 Three patients will be entered at each dose level with 6 patients entered at the maximum tolerated or maximum attainable dose (limitation imposed by production of the adenovirus).
- 5.4 Dose Escalation: The adenovirus dose will increase in one-log<sub>10</sub> increments for each group until 10<sup>9</sup> PFUs are reached. Patients entered at a given dose level will not be eligible for dose escalation. We estimate based on the achievable titers of adenovirus that six dose levels can be done thus requiring 21 patients in each group (resectable and non-resectable).
- 5.5 All patients shall be registered with the Data Management office at \_\_\_\_\_ At the time of registration, a prestudy form shall be necessary on each patient. When applicable, information pertaining to important prognostic factors such as tumor histology, pretreatment weight loss, performance status, disease stage and extent, and prior therapy will be requested.
- 5.6 Patients with local-regional tumors will have injection of a total dose of 10 ml for tumors  $\geq 4$  cm in diameter or 3 ml for tumor  $< 4$  cm in diameter of the adenovirus preparation with the appropriate number of viral particles at multiple sites percutaneously or transorally. Direct endoscopic injections may also be used is required. Injections will be placed at approximately 1 centimeter increments.
- 5.7 The treatment will be repeated three times weekly for two weeks. Dose escalation may proceed after a minimum two week follow-up of the last patient entered into the previous dose level. Treatment will continue on a monthly basis as long as there is no tumor progression. After one year the patients will be evaluated for continuation of therapy.
- 5.8 Patients will wear a surgical mask for 24 hours following injection of the adenovirus. All medical personnel will wear masks and face shields routinely during endoscopy and injection of the adenovirus. Anti-tissves will be prescribed as necessary. All patients will be kept in isolation during the time they are receiving injections of the adenovirus and for 48 hours after the last injection.
- 5.9 Those patients with surgically resectable disease will be treated by tumoral injection of adenovirus preparation as described in 5.6 and 5.7. The treatment will be repeated for two consecutive courses. Within four days of completion of the second course, the patients will be eligible to proceed with surgical resection. At the completion of surgical resection, prior to closure, 10ml of adenovirus preparation will be administered into the surgical defect (operative bed) and allowed to remain in contact for 60 minutes. The wounds are then closed and drains placed. Post-operatively, on the third post-operative day (prior to drain removal), 10ml of adenovirus preparation is sterily introduced into the drains and retrograde placed into the wounds and is allowed to remain for two hours. The drains are then replaced to suction and removed when indicated by the attending staff surgeon.

#### PRE-TREATMENT EVALUATION

- 6.1 A complete history and physical to include performance status, recent weight loss, usual weight and concurrent non-malignant disease and its therapy, and all prior anticancer treatments must be recorded.
- 6.2 Laboratory studies shall include quantitative immunoglobulins; a CBC with differential and platelet count; SMA-12 and electrolytes, including creatinine, bilirubin, SGPT, alkaline phosphatase, HIV analysis, urinalysis, and chest x-ray.
- 6.3 Any residual toxicity from prior therapies should be recorded using the grading schema in Appendix C.
- 6.4 Appropriate studies should be obtained to fully define the extent and severity of existing or suspected malignant and non-malignant disease.
- 6.5 Measurements of disease that can be measured on a C.T. scan of the head and neck will be documented. The location and size must be recorded prior to treatment. A photograph of the area

The University of Texas  
M. D. ANDERSON  
CANCER CENTER

MEMORANDUM

DATE: October 4, 1995

TO: Dr. Gary Clayman  
Department of Head and Neck Surgery

FROM: Myriam Brena *Myriam Brena*  
Secretary, Surveillance Committee (IRB)  
Office of Protocol Research

SUBJECT: Administrative Approval of Revised Protocol HNS 94-001\*, entitled  
"Clinical Protocol for Modification of Tumor Suppressor Gene Expression  
in Head and Neck Squamous Cell Carcinoma (HNSCC) with an  
Adenovirus Vector Expressing Wildtype p53"

Official Approval Date: 10/2/95

Dr. Aman U. Buzdar, Surveillance Committee Chairman, reviewed and administratively approved the revision of the above named and numbered protocol.

This approval does not alter or otherwise change the annual review date of this protocol.

\* Revision date 9/25/95

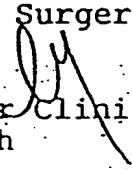
c: Leonard A. Zwelling, M.D.



THE UNIVERSITY OF TEXAS  
MD ANDERSON  
CANCER CENTER

MEMORANDUM

TO: Gary Clayman, M.D.  
Department of Head and Neck Surgery

FROM: Leonard A. Zwelling, M.D.   
Associate Vice President for Clinical  
and Translational Research

DATE: October 10, 1995

SUBJECT: Activation and Distribution of New Protocol HNS 94-001

Attached find a copy of the above referenced protocol. This study is now ready for patient accrual. Should you have any questions, please do not hesitate to contact me.

Please distribute a copy of this protocol to the appropriate individuals in your department/section and collaborators.

LAZ/lmb  
attachment

14

**SURVEILLANCE COMMITTEE (IRB)**  
**ANNUAL REVIEW OF CLINICAL, LABORATORY AND MISCELLANEOUS PROTOCOLS**

**REPORT DATE:**

**DUE DATE:**

08/31/95

The annual review process is a requirement of this institution in compliance with federal regulations. The Study Chairperson is responsible for updating the protocol status each year by providing the following information to the Surveillance Committee (BOX 38) for potential approval by the due date shown above. Direct your questions to Myriam Brena, Secretary, Surveillance Committee (IRB) at extension 2-2933.

**Protocol Number and Title:** HNS 94-001 - "Clinical Protocol for Modification of Tumor Suppressor Gene Expression in Head and Neck Squamous Cell Carcinoma (HNSCC) with an Adenovirus Vector Expressing Wildtype p53"

**Study Chairperson:** Dr. Gary Clayman  
Department of Head and Neck Surgery

**For Committee Review on:** 09/20/95  
**Last Annual Review:** / /

Does the protocol involve drugs or devices obtained via an Investigational New Drug Exemption (IND) or Investigational Device Exemption (IDE)? ☐ Yes ☐ No Number \_\_\_\_\_

Does this study include any products manufactured or produced at MDACC? ☐ Yes ☐ No

If yes, identify the room where the product is manufactured. \_\_\_\_\_

**Current Protocol Status (Date)**

Original IRB Approval

Informed Consent (last revision) Please attach the consent form with the latest revision of 2/16/94

Not Yet Activated (i.e. pending approval, drug, etc., but anticipated future activation).

(NOTE: Justification is required for any study not activated within one year (12 months) of IRB approval.

Active (study in progress and accruing patients).

Closed To New Patient Entry (no new patient accrual, but patients continuing on treatment or still alive for follow up).

Date last patient entered \_\_\_\_\_

Terminated (no activity - all patients off study). NOTE: A final report is required.

Withdrawn (study never activated, no patient registrations. No future activity is anticipated)

Maximum number of patients or samples approved 42

Total accrual to date 0

Estimated number of additional patients or samples required to complete study \_\_\_\_\_

Have any recent reports of preliminary analyses been prepared since the last annual review? ☐ Yes ☐ No

If yes, provide a copy.

Total ADRs reported ✓ ADRs since last review \_\_\_\_\_ Number of treatment related deaths \_\_\_\_\_

Please provide brief synopsis of status of study including response and toxicity information (use additional pages if required)

Awaiting NIH/IRAC approval prior to study  
commencing. Presently FDA approved

Study Chairman (Signature and Date)

Division Head or Department Chairman (Signature and Date)

Surveillance Committee Comments \_\_\_\_\_

Surveillance Committee Chairman or Designee (Signature and Date)

Rev. 8/84

☒ Approved

☐ Deferred

☐ Approved Contingent



**MEMORADUM**

**DATE:** July 29, 1997

**TO:** Elias Dayam  
Investigational Pharmacy  
M.D. Anderson Cancer Center

**FROM:** Amber Hutchison  
Sr. Clinical Research Associate *UH*

**PROTOCOL:** INT-002 (HNS 94-001)

**RE:** Study Close-out Drug Accountability and Material Return

**Cc:** Gary Clayman, M.D.  
Patty Bruso  
Jay Merritt, M.D.  
Dee Connors  
Study file

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Enrollment and treatment of patients in the above clinical study has been completed. According to the Audit Report sent to me on July 28, 1997 there is one remaining vial in the freezer.

Attached is a Material Return and Disposition Record to be completed and returned with the shipment of one (1) vial of Lot 0057-0003 Part 8.50013A ( $2 \times 10^{10}$  PFU). This shipment should be sent to the attention of

If you have any questions, please do not hesitate to contact me at  
pager at

; or by

## Inhibition of Viral and Cellular Promoters by Human Wild-Type p53

M. A. SUBLER, D. W. MARTIN, AND S. DEB\*

*Department of Microbiology, University of Texas Health Science Center, San Antonio, Texas 78284-7758*

Received 20 March 1992/Accepted 1 May 1992

Mutation of the p53 tumor suppressor gene is a recurring event in a variety of human cancers. Wild-type p53 may regulate cell proliferation and has recently been shown to repress transcription from several cellular promoters. We studied the effects of wild-type and mutant human p53 on the human proliferating-cell nuclear antigen promoter and on several viral promoters including the simian virus 40 early promoter-enhancer, the herpes simplex virus type 1 thymidine kinase and UL9 promoters, the human cytomegalovirus major immediate-early promoter-enhancer, and the long terminal repeat promoters of Rous sarcoma virus, human immunodeficiency virus type 1, and human T-cell lymphotropic virus type I. HeLa cells were cotransfected with a wild-type or mutant p53 expression vector and plasmids containing a chloramphenicol acetyltransferase reporter gene under viral (or cellular) promoter control. Expression of wild-type p53 correlated with a consistent and significant (6- to 76-fold) reduction of reporter enzyme activity. A mutation at amino acid 143 of p53 releases this inhibition significantly with all the promoters studied. Expression of a p53 mutated at any one of the five amino acid positions 143, 175, 248, 273, and 281 also correlated with a much smaller (one- to sixfold) reduction of reporter enzyme activity from the herpes simplex virus type 1 thymidine kinase promoter. These mutant forms of p53 are found in various cancer cells. Thus, failure of tumor suppression correlates with loss of the promoter inhibitory effect of p53.

p53 is a nuclear phosphoprotein that was initially detected in association with simian virus 40 (SV40) large T antigen in virus-transformed rodent cells (31, 33). Elevated levels of p53 were subsequently observed in cell lines transformed by a variety of agents, including DNA and RNA tumor viruses, irradiation, and chemical carcinogens (13, 16, 24, 29, 35, 48). When genomic and cDNA clones of p53 were found to immortalize primary cells and to cooperate with the *ras* oncogene in transformation of primary cells, p53 was assigned to the nuclear oncogene family of *myc* and *myb* (16, 29, 45); only recently has it been learned that the original clones contained activating mutations (24). Expression of wild-type p53 has now been shown to inhibit proliferation of transformed cells, suppress oncogene-mediated cell transformation, and eliminate the tumorigenic potential of tumor-derived cell lines (2, 3, 8, 9, 14-16, 18, 36, 38, 40). Like the retinoblastoma susceptibility (RB) gene, p53 is now considered to be an antioncogene or tumor suppressor gene (see reference 32 for a review). Somatic and germ line (in Li-Fraumeni syndrome) mutation of the p53 gene has been detected in a variety of human tumors, with mutations concentrated in phylogenetically conserved sequence domains (26, 32, 34, 54). At present, p53 mutations are the most frequently reported genetic defects in human cancer (3, 26, 27, 44, 56, 58).

Several biochemical functions are attributed to p53. p53-GAL4 fusion proteins can activate transcription from promoters containing GAL4-binding sites, suggesting that p53 is a transactivator (17, 47). Moreover, sequence-specific DNA binding by p53 has been reported (4, 30). Wild-type (but not mutant) p53 binds to the 21-bp repeats of the SV40 early and late promoters (4) and to TGCCCT repeats present in the human ribosomal gene cluster (30). p53 inhibits SV40 DNA replication in vivo and in vitro by complexing with T antigen

and inhibiting the unwinding capability of T antigen (5, 19, 20, 59). Wild-type p53 has recently been shown to inhibit *c-fos* transcription (21) and to repress transcription from several cellular promoters (10, 21, 50).

The human proliferating-cell nuclear antigen (PCNA) gene is growth regulated (1, 28) and encodes a protein that is a component of the DNA replication machinery of the cell. PCNA has been identified as a cofactor of DNA polymerase  $\delta$  (6, 46, 55). Mercer et al. (39) demonstrated a down-regulation of PCNA mRNA and protein by wild-type p53. However, the mechanism of this regulation was not known since the possibility that p53 might affect the PCNA promoter activity was not examined.

Several viruses have mechanisms to target (and presumably inactivate) wild-type p53 by their transforming proteins. SV40 large T antigen, adenovirus 5 E1B, and E6 of human papillomavirus (HPV) types 16 and 18 bind specifically to p53 and either sequester it (large T, E1B) or promote its degradation (E6) (31, 33, 51, 52, 60). The effect of p53 on promoter activity of viruses has not been investigated in detail.

We studied the effect of wild-type and mutant human p53 expression on the activity of PCNA and several viral promoters fused to a chloramphenicol acetyltransferase (CAT) reporter gene. Expression of wild-type p53 correlated with a consistent and significant (6- to 76-fold) inhibition of reporter enzyme activity in HeLa cells. Significantly, mutants of p53 found in cancer cell lines exert this inhibitory effect on the promoter function in this assay. This suggests that the promoter inhibitory activity of p53 is crucial for its tumor suppressor activity.

### MATERIALS AND METHODS

**DNA plasmids.** Wild-type and mutant human p53 expression plasmids (generously provided by Arnold J. Levine) utilize the human cytomegalovirus (HCMV) major immedi-

\* Corresponding author.

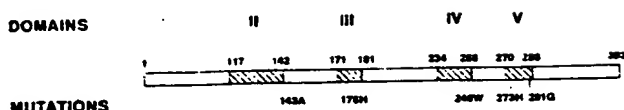


FIG. 1. Schematic representation of the p53 gene product. Conserved domains II to V are indicated by hatched areas. Positions of amino acid substitutions in the mutants that are used in this study are indicated below.

ate-early promoter-enhancer (-671 to +73) in the vector pHCMV-Neo-Bam (25). p53-cWT contains a wild-type p53 cDNA, while p53-c143A (Val to Ala at amino acid 143) and p53-c248W (Arg to Trp at amino acid 248) contain mutant p53 cDNAs (25). p53-175H (Arg to His at amino acid 175), p53-273H (Arg to His at amino acid 273), and p53-281G (Asp to Gly at amino acid 281) are mutant p53 cDNA-genomic chimeras, all containing introns 2 through 4 (25). The neomycin resistance gene was removed from all plasmids by treatment with *HindIII* and *XbaI*.

The CAT plasmids described here all contain the *Escherichia coli* CAT gene under the transcriptional control of the following promoters: PCNA (human PCNA promoter) (41); pSV2 (SV40 early promoter-enhancer) (22); CMV (HCMV major immediate-early promoter-enhancer) (11); HSV-1.TK (herpes simplex virus type 1 thymidine kinase promoter) (37); UL9 (HSV-1 UL9 gene promoter [12a]); RSV (Rous sarcoma virus 3' long terminal repeat [LTR]) (12); HIV-1 (human immunodeficiency virus type 1 LTR) (43); and HTLV-I (human T-cell lymphotropic virus type I LTR) (53). The plasmids are designated promoter name.CAT. PCNA.CAT was generously provided by Gilbert Morris.

**Cell culture and transfection.** Human cervical carcinoma (HeLa) and monkey kidney (Vero) cells were obtained from the American Type Culture Collection and propagated in minimum essential medium containing 10% fetal calf serum and Dulbecco's minimum essential medium, respectively. Subconfluent cells were transfected by the calcium phosphate-DNA coprecipitation method with a dimethyl sulfoxide shock 4 h posttransfection (8, 23). In a typical experiment,  $5 \times 10^6$  cells were cotransfected with 2.5  $\mu$ g of a reporter gene construct and 5  $\mu$ g of a p53 expression plasmid (or 5  $\mu$ g of the expression vector without p53 sequences as a control). All transfection experiments were repeated several times.

**CAT assay.** Cells were harvested 48 h posttransfection and lysed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT enzyme activity (22). CAT activity was detected by thin-layer chromatographic separation of [ $^{14}$ C]chloramphenicol from its acetylated derivatives and quantitated by cutting out radioactive spots from the thin-layer chromatograph plate after autoradiography.

**Metabolic labeling and immunoprecipitation.** At 48 h after transfection with 20  $\mu$ g of wild-type or mutant human p53 expression plasmids (or expression vector pHCMV-Bam), HeLa cells were incubated in methionine-free minimal essential medium for 20 min and subsequently metabolically labeled for 4 h with [ $^{35}$ S]methionine (ICN Tran[ $^{35}$ S]-label) at 70  $\mu$ Ci/ml in methionine-free minimal essential medium (49). Cells were lysed, and extract aliquots were immunoprecipitated with PAb421, a cross-species, carboxy-terminal-specific, anti-p53 monoclonal antibody (p53 Ab-1; Oncogene Science) (57), and protein A-agarose (Calbiochem). Immunoprecipitated proteins were separated by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

## RESULTS

**Expression of wild-type and mutant human p53 proteins in transfected HeLa cells.** We used wild-type and mutant human p53-expressing clones for our analysis of the effect(s) of p53 on the function of various promoters. The mutants were c143A, 175H, c248W, 273H, and 281G, where capital letters indicate mutant amino acids and small c indicates cDNA clones. These mutants were chosen because they contain the frequently mutated amino acid residues found in tumors (26) (Fig. 1). These residues fall in or near four domains (II to V) which are highly conserved in vertebrate species (54).

To determine whether the mutants and wild-type proteins were expressed, we transfected HeLa cells with wild-type or mutant p53 expression plasmids or with expression vector pHCMV.Bam (vector alone) and metabolically labeled them with [ $^{35}$ S]methionine. Cell lysates were immunoprecipitated with PAb421, a cross-species, carboxy-terminal-specific, anti-p53 monoclonal antibody, and protein A-agarose. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 2). Transfection of HeLa cells with either wild-type or mutant p53 expression plasmids led to the specific immunoprecipitation of proteins migrating at an approximate molecular weight of 53,000 (indicated by an arrowhead), while transfection with the expression vector did not. Mutant p53 proteins appear to be expressed at higher levels than wild-type in transfected HeLa cells. This is consistent with the extended half-life of mutant p53 proteins (32). This effect would also be due to inhibitory effects exerted by wild-type p53 on the CMV promoter. Mutant proteins would not be as inhibitory, resulting in a higher level of expression. In the lanes containing mutant p53s (c143A, 175H, and 281G), a band at about 70 kDa is visible. This band may indicate complex formation between mutant p53s and the cellular heat shock protein 70 (25, 32). We do not know the identity of the 18-, 43-, and 200-kDa bands seen in all lanes, including vector alone. The immunoprecipitation results shown in Fig. 2 clearly indicate successful expression of wild-type and mutant human p53s in HeLa cells after transfection with the corresponding expression plasmids.

**Modulation of PCNA promoter activity by wild-type and**

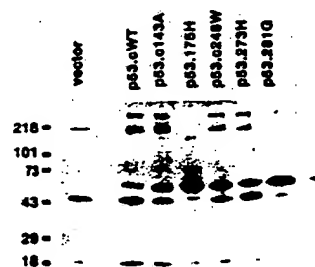


FIG. 2. Expression of wild-type and mutants of human p53 by transfection of expression plasmids into HeLa cells. HeLa cells were transfected with pHCMV.Bam expression vector alone and wild-type or mutant human p53 expression plasmid DNA; the proteins were then metabolically labeled with [ $^{35}$ S]methionine and immunoprecipitated with p53-specific monoclonal antibody as described in Materials and Methods. Immunoprecipitates were analyzed on an SDS-polyacrylamide gel. The arrowhead shows bands corresponding to p53. Numbers on left show sizes in kilodaltons.

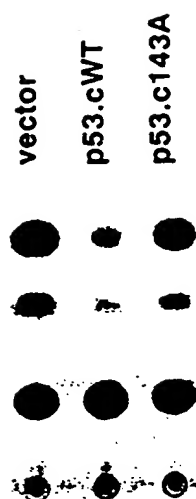


FIG. 3. Effect of wild-type and a mutant human p53 on the expression of PCNA.CAT in HeLa cells. Subconfluent HeLa cells were cotransfected with PCNA.CAT (2.5  $\mu$ g) and pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type or a mutant p53 (143 V→A), using the calcium phosphate precipitation technique as described in Materials and Methods. At 48 h posttransfection, cells were harvested and a CAT assay was done as described in the text. Experiments were repeated several times with similar qualitative results; one representative example is shown.

mutant human p53. The PCNA gene encodes a nuclear protein that acts as an auxiliary factor of DNA polymerase  $\delta$  and is presumably a part of the cellular replication machinery (55). It has been shown previously (39) that growth suppression induced by wild-type p53 protein is accompanied by a down-regulation of PCNA expression. Therefore, we were interested in determining whether wild-type p53 can inhibit the function of the PCNA promoter and, if so, whether a mutant p53 can exert the same effect. PCNA.CAT (41) was cotransfected into HeLa cells by the calcium phosphate precipitation technique as described in Materials and Methods with the pHCMV.Bam expression vector alone or with the plasmid expressing either the wild-type or the mutant (c143A) form of p53. After 48 h, CAT activity (PCNA promoter activity) was assayed in these cells. Wild-type p53 inhibited PCNA.CAT activity in transient assays by more than sixfold, while the mutant inhibited activity by one- to twofold (Fig. 3). Thus, the promoter inhibition is due to wild-type p53, and a mutation in the p53 gene destroys the inhibitory effect.

**Effect of expression of wild-type and mutant p53 on various viral promoters.** To analyze the effect of expression of wild-type and mutant p53 on various viral promoters, we used the following promoter-CAT constructs: SV40 early promoter (pSV2.CAT) (22), CMV early promoter-enhancer (CMV.CAT) (11), HSV-1 UL9 promoter (UL9.CAT) (13a), HIV-1 LTR (HIV.CAT) (43), RSV LTR (RSV.CAT) (12), and HTLV-1 LTR (HTLV.CAT) (53). The promoter activities were determined by CAT assay after cotransfecting the respective promoter constructs with the pHCMV.Bam expression vector alone or with the plasmid expressing either the wild-type or a mutant (c143A) form of p53 into HeLa cells (Fig. 4; Table 1). The experiments were repeated several times with qualitatively similar results. Representative examples are shown in Fig. 4. All the promoters were inhibited significantly

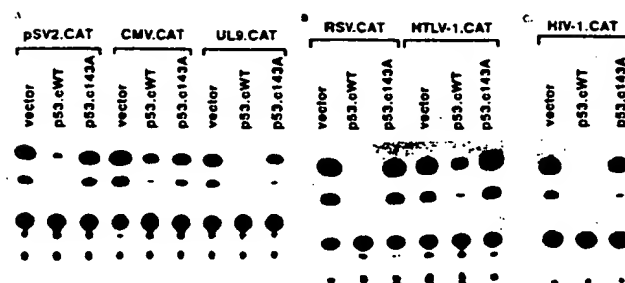


FIG. 4. Effect of wild-type and a mutant human p53 on the expression of viral promoter-CAT constructs in HeLa cells. The promoter-CAT constructs indicated (see text) were transfected separately into HeLa cells along with pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type or mutant p53 (143 V→A) as described in the legend to Fig. 3. For pSV2.CAT, 0.5  $\mu$ g of the CAT plasmid was used with 5  $\mu$ g of vector or p53 expression plasmid. All others were used as described in Materials and Methods.

by the expression of wild-type p53. On the other hand, the mutant p53 had a relatively minor, if any, effect on expression of the various promoter-CAT constructs. In most of the cases, although the inhibition persisted with the mutant p53 (c143A), its extent was greatly reduced. In at least one case (HTLV-1.CAT), the mutant actually stimulated the activity about 50%. This is not entirely surprising since recently Chin et al. (10) reported that the human multi-drug-resistant (*MDR1*) gene promoter is activated by another mutant p53 (175H).

All the promoters examined were inhibited by wild-type human p53, albeit to different extents. SV40 early promoter seems to be least affected under our assay conditions. To observe a significant extent of inhibition, we had to lower the pSV2.CAT concentration to 0.5  $\mu$ g per transfection. The difference in the extent of inhibition by the same amount of wild-type p53 expression construct indicates that the observed promoter inhibition is possibly not an effect of general lethality caused by p53.

**Effect of expression of wild-type and various mutants of human p53 on HSV-1 TK gene promoter.** To determine the effect of other mutant p53 proteins on promoter activity, we tested wild-type and various mutants of human p53 with the HSV-1 TK gene promoter-CAT construct TK.CAT. The mutants of human p53 chosen for this study were described above and are as follows: p53-c143A, p53-175H, p53-c248W, p53-273H, and p53-281G. As shown in Fig. 5, TK promoter activity was inhibited most dramatically by wild-type p53, while the mutants inhibited to different extents. It is clear

TABLE 1. Inhibition of activity of different promoters by human wild-type (WT) p53 and mutant c143A relative to vector alone in HeLa cells

Promoter	Activity relative to vector alone (%)	
	WT p53	c143A p53
PCNA	15.9	58.8
RSV LTR	1.3	76.9
HTLV-1 LTR	14.3	156.3
HIV LTR	2.3	47.6
UL9 (HSV)	2.8	31.2
SV40 early promoter	6.1	90.9
CMV early promoter	7.2	16.1

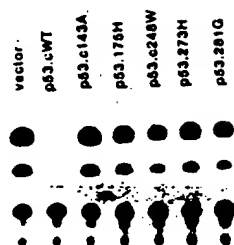


FIG. 5. Effect of expression of different mutant human p53s on the expression of HSV-1 TK promoter activity. HeLa cells were cotransfected with TK.CAT and pHCMV.Bam (vector alone) or pHCMV.Bam expressing either wild-type (cWT) or one of the mutant p53s: c143A (V to A at amino acid 143), 175H (R to H at amino acid 175), c248W (R to W at amino acid 248), 273H (R to H at amino acid 273), and 281G (D to G at amino acid 281) as described in the text. CAT assays were done as described in the text.

that all the mutants tested show a dramatic loss in mediating inhibition of the promoter. The failure of tumor suppression by these mutant p53 proteins correlates with the loss of the promoter inhibitory effect.

**Inhibition of activity of various promoters by wild-type human p53 in Vero cells.** To determine whether the p53-mediated promoter inhibition is cell type specific or is influenced by the expression of E6 of HPV 18 in the HeLa cell line, we chose also to use a monkey kidney cell line (Vero). Table 2 shows the percentage of acetylation of [<sup>14</sup>C]chloramphenicol with various promoters in the presence and absence of wild-type human p53. The results indicate that in the Vero cell line (a nontransformed cell line) also, wild-type human p53 significantly inhibits various promoter activities (6- to 28-fold).

## DISCUSSION

The results described above show that overexpression of wild-type human p53 can exert an inhibitory effect on a variety of viral promoters as well as on the cellular PCNA promoter (6- to 76-fold, Table 1). Several other groups recently reported an inhibitory activity of p53 on different cellular promoters. Santhanam et al. (50) found that wild-type p53 inhibited the promoters for interleukin 6, *c-fos*, *beta-actin*, and the porcine major histocompatibility complex class I gene. Ginsberg et al. (21) described the inhibition of *c-fos*, *beta-actin*, p53, *hsc70*, and *c-jun* promoters, while Chin et al. (10) showed that the *MDR1* gene promoter was inhibited by p53. Combining our results with those reported previously, it becomes clear that a wide variety of cellular and viral promoters are inhibited by wild-type human p53. In all the cases, mutant p53 proteins found in tumors were either less inhibitory or in some cases stimulatory (10) (Fig.

TABLE 2. Inhibition of different promoters by human wild-type p53 relative to vector alone in Vero cells

Promoter	Activity relative to vector alone (%)	Fold inhibition
PCNA	15.4	6.5
RSV LTR	3.5	28.6
HIV LTR	10.3	9.7
UL9 (HSV)	11.1	9.0
CMV	12.5	8.0

TABLE 3. Inhibition of HSV-1 TK promoter by human wild-type p53 and different mutants

p53	Activity relative to vector alone (%)
cWT	1.1
c143A	66.7
175H	37.0
c248W	14.5
273H	37.0
281G	21.2

4) for the promoter function. The extent of inhibition by wild-type p53 appears to depend on the promoter tested (Table 1); for example, the CMV promoter (CMV.CAT) was not inhibited to the same extent as the HIV LTR promoter (HIV.CAT). The SV40 early promoter seems to be least affected under our assay conditions. To observe a significant extent of inhibition, we had to lower the pSV2.CAT concentration to 0.5 µg per transfection. The difference in the extent of inhibition by the same amount of wild-type p53-expressing construct indicates that the observed promoter inhibition is possibly not an effect of general lethality caused by p53. Also, different mutants of p53 have different quantitative effects on promoter inhibition (Fig. 5; Table 3). Despite these differences, it is both interesting and significant that such a wide variety of viral promoters are inhibited by p53. What effect endogenous p53 has on these promoters in the course of viral infection is not known. It remains to be seen whether all these viruses may have molecular mechanisms to circumvent p53 inhibition. This may represent a unique strategy to allow viral replication not previously defined for nononcogenic viruses, whereas alteration of p53 by tumor viruses such as SV40, adenovirus, and HPV 16 and 18 has been established (31, 33, 51, 52, 60).

Most of our experiments were performed in HeLa cells, which are known to have the E6 protein of HPV 18. Since HPV 18 E6 protein is known to interact with p53, a possibility remains that the data observed were influenced by this interaction. However, we also observed significant promoter inhibition in Vero cells (a nontransformed cell line) (Table 2). This suggests that the promoter inhibition is probably because of p53 alone.

Because p53 possesses the promoter inhibitory activity, it is possible that at least one of the mechanisms by which wild-type p53 inhibits cellular proliferation is by inhibiting cellular promoters. This is based on the assumption that p53 directly inhibits transcriptional activity. This remains to be determined by using *in vitro* transcription systems. However, the fact that the inhibitory effect of p53 is exerted on a wide variety of promoters, both cellular and viral, suggests that p53 probably affects one or more of the common generalized transcription factors or that it binds to promoter sequences nonspecifically and inhibits transcription. At this stage, we should also be aware of the possibility that the observed promoter inhibition is an effect of wild-type p53 when it is overexpressed. Under normal conditions, such a high concentration of p53 is not expected. However, it is not difficult to imagine that at a certain point in the cell cycle, local effective concentration of p53 may rise high enough to modulate cellular promoter activities.

p53 remains an extremely important and intriguing molecule. It has been demonstrated that purified wild-type p53 can bind to cellular DNA (30) as well as the SV40 early promoter region (4). It has also been shown that it can

function as a transcriptional activator when expressed as a chimera with the GAL4 DNA-binding domain on promoters with GAL4 DNA-binding sites (17, 47). The same molecule may function as an activator as well as an inhibitor of transcription. It is tempting to speculate that while p53 acts as a generalized inhibitor of transcription, it could activate certain promoters where it can bind effectively. We have observed that it requires a relatively higher concentration of p53 plasmid to inhibit the SV40 early promoter, which has p53-binding sites (4). One can speculate that p53 may exert its tumor suppressor function in several ways. First, under certain conditions, p53 may inhibit genes required for progression through the cell cycle. The inhibition of the PCNA promoter demonstrated in this study supports this mechanism. In addition, p53 may also activate expression of genes involved in the regulation of normal cell cycle progression. This regulation of expression may require the presence of p53-binding sites as *cis*-acting factors at the target gene. A possibility also exists that p53 activates the production of a factor that interacts with the transcription machinery and inhibits gene expression. Thus, p53 may act as a central factor in controlling the dynamic pattern of gene expression required for maintenance of a normal cell cycle. Both the tumor suppressor gene products RB and p53 have cellular antiproliferative activity. In one way, at least, they have a similar biochemical function—inhibition of transcription. It has been suggested that at least one mechanism by which RB may inhibit specific transcriptional activity is by complexing with the transcription factor E2F (7, 42). While it is not yet clear how p53 exerts its effect, similarity in biochemical function is an intriguing common theme.

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#### REFERENCES

- Almendral, J. M., D. Huebsch, A. P. Blundell, H. MacDonald-Bravo, and R. Bravo. 1987. Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. *Proc. Natl. Acad. Sci. USA* 84:1575-1579.
- Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. Van Tuizen, D. H. Ledbetter, D. F. Barker, Y. Nakamura, R. White, and B. Vogelstein. 1989. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* 244:217-221.
- Baker, S. J., K. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 249:912-915.
- Bargonetti, J., P. N. Friedman, S. E. Kern, B. Vogelstein, and C. Prives. 1991. Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. *Cell* 65:1083-1091.
- Braithwaite, A. W., H. W. Sturzbecher, C. Addison, C. Palmer, K. Rudge, and J. R. Jenkins. 1987. Mouse p53 inhibits SV40 origin-dependent DNA replication. *Nature (London)* 329:458-460.
- Bravo, R., R. Frank, A. P. Blundell, and H. MacDonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase  $\delta$ . *Nature (London)* 326:515-517.
- Cao, L., B. Faba, M. Dembski, L.-H. Tsai, E. Harlow, and N. Dyson. 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. *Nature (London)* 355:176-179.
- Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745-2752.
- Chen, P.-L., Y. Chen, R. Bookstein, and W.-H. Lee. 1990. Genetic mechanisms of tumor suppression by the human p53 gene. *Science* 250:1576-1580.
- Chin, K.-V., K. Ueda, I. Pastan, and M. M. Gottesman. 1992. Modulation of activity of the promoter of the human *MDR1* gene by *ras* and p53. *Science* 255:459-462.
- Cullen, B. R. 1986. Trans-activation of human immunodeficiency virus occurs via a bimodal mechanism. *Cell* 46:973-982.
- Cullen, B. R., P. T. Lomedico, and G. Ju. 1984. Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukaemogenesis. *Nature (London)* 307:241-245.
- Deb, S. P. Unpublished data.
- DeLeo, A. B., G. Jay, E. Appella, G. C. Dubois, L. W. Law, and L. J. Old. 1979. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc. Natl. Acad. Sci. USA* 76:2420-2424.
- Diller, L., J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressan, M. Ozturk, S. J. Baker, B. Vogelstein, and S. H. Friend. 1990. p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.* 10:5772-5781.
- Eliyahu, D., D. Michalovitz, S. Eliyahu, O. Pinhasi-Kimhi, and M. Oren. 1989. Wild-type p53 can inhibit oncogene-mediated focus formation. *Proc. Natl. Acad. Sci. USA* 86:8763-8767.
- Eliyahu, D., A. Raz, P. Gruss, D. Givol, and M. Oren. 1984. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. *Nature (London)* 312:646-649.
- Fields, S., and S. K. Jang. 1990. Presence of a potent transcription activating sequence in the p53 protein. *Science* 249:1046-1049.
- Finlay, C. A., P. W. Hinds, and A. J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell* 57:1083-1093.
- Friedman, P. N., S. E. Kern, B. Vogelstein, and C. Prives. 1990. Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. *Proc. Natl. Acad. Sci. USA* 87:9275-9279.
- Gannon, J. V., and D. P. Lane. 1987. p53 and DNA polymerase  $\alpha$  compete for binding to T antigen. *Nature (London)* 329:456-458.
- Ginsberg, D., F. Mechta, M. Yaniv, and M. Oren. 1991. Wild-type p53 can down-modulate the activity of various promoters. *Proc. Natl. Acad. Sci. USA* 88:9979-9983.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
- Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
- Hinds, P., C. Finlay, and A. J. Levine. 1989. Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. *J. Virol.* 63:739-746.
- Hinds, P. W., C. A. Finlay, R. S. Quartin, S. J. Baker, E. R. Fearon, B. Vogelstein, and A. J. Levine. 1990. Mutant p53 DNA clones from human colon carcinomas cooperate with *ras* in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. *Cell Growth Differ.* 1:571-580.
- Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris. 1991. p53 mutations in human cancers. *Science* 253:49-53.
- Iggo, R., K. Gatter, J. Bartek, D. Lane, and A. L. Harris. 1990.



- Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* 335:675-679.
28. Jaskulski, D., C. Gatti, S. Travali, B. Calabretta, and R. Baserga. 1988. Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. *J. Biol. Chem.* 263:10175-10179.
  29. Jenkins, J. R., K. Rudge, and G. A. Currie. 1984. Cellular immortalization by a cDNA clone encoding the transformation associated phosphoprotein p53. *Nature (London)* 312:651-653.
  30. Kera, S. E., K. W. Kinzler, A. Bruskina, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein. 1991. Identification of p53 as a sequence-specific DNA-binding protein. *Science* 252:1708-1711.
  31. Lane, D. P., and L. W. Crawford. 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature (London)* 278:261-263.
  32. Levine, A. J., J. Momand, and C. A. Finlay. 1991. The p53 tumor suppressor gene. *Nature (London)* 351:453-456.
  33. Linzer, D. I. H., and A. J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40 transformed cells and uninfected embryonal carcinoma cells. *Cell* 17:43-52.
  34. Malkin, D., F. P. Li, L. C. Strong, J. F. Fraumeni, C. E. Nelson, D. H. Kim, J. Kassel, M. Gryka, F. Z. Bischoff, M. A. Tainsky, and S. H. Friend. 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* 250:1233-1238.
  35. Maltzman, W., M. Oren, and A. J. Levine. 1981. The structural relationships between 54,000 molecular weight cellular tumor antigens detected in viral and nonviral transformed cells. *Virology* 112:145-156.
  36. Martinez, J., I. Georgoff, J. Martinez, and A. J. Levine. 1991. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. *Genes Dev.* 5:151-159.
  37. McKnight, S. L., and R. Kingsbury. 1982. Transcriptional control signals of a eukaryotic protein-coding gene. *Science* 217:316-324.
  38. Mercer, W. E., M. Amin, G. J. Sauve, E. Appella, S. J. Ullrich, and J. W. Romano. 1990. Wild-type human p53 is antiproliferative in SV40-transformed hamster cells. *Oncogene* 5:973-980.
  39. Mercer, W. E., M. T. Shields, D. Lin, E. Appella, and S. J. Ullrich. 1991. Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen expression. *Proc. Natl. Acad. Sci. USA* 88:1958-1962.
  40. Michalivitz, D., O. Halevy, and M. Oren. 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* 62:671-680.
  41. Morris, G. F., and M. B. Mathews. 1990. Analysis of the proliferating cell nuclear antigen promoter and its response to adenovirus early region 1. *J. Biol. Chem.* 265:16116-16125.
  42. Mudryj, M., S. H. Devoto, S. W. Hiebert, T. Hunter, J. Pines, and J. R. Nevins. 1991. Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. *Cell* 65:1243-1253.
  43. Nabel, G. J., S. A. Rice, D. M. Knipe, and D. Baltimore. 1987. Alternative mechanisms for activation of human immunodeficiency virus enhancer in T cells. *Science* 239:1299-1302.
  44. Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Clearly, S. H. Bigner, N. Davidson, S. Baylin, P. Devillee, T. Glover, F. S. Collins, A. Weston, R. Modali, C. C. Harris, and B. Vogelstein. 1989. Mutations in the p53 gene occur in diverse human tumor types. *Nature (London)* 342:705-708.
  45. Parada, L. F., H. Land, R. A. Weinberg, D. Wolf, and V. Rotter. 1984. Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation. *Nature (London)* 312:649-651.
  46. Prelich, G., C. K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman. 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase- $\delta$  auxiliary protein. *Nature (London)* 326:517-520.
  47. Raycroft, L., H. Wu, and G. Lozano. 1990. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. *Science* 249:1049-1051.
  48. Rotter, V., M. A. Boss, and D. Baltimore. 1981. Increased concentrations of an apparently identical cellular protein in cells transformed by either Abelson murine leukemia virus or other transforming agents. *J. Virol.* 38:336-346.
  49. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1990. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
  50. Santhanam, V., A. Ray, and P. Sehgal. 1991. Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product. *Proc. Natl. Acad. Sci. USA* 88:7605-7609.
  51. Sarnow, P., Y. Ho, J. Williams, and A. J. Levine. 1982. The adenovirus E1b-58K tumor antigen and SV40 large tumor antigen are physically associated with the same 54K cellular protein. *Cell* 28:387-394.
  52. Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63:1129-1136.
  53. Sodroski, J. G., C. A. Rosen, and W. A. Haseltine. 1984. Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. *Science* 225:381-385.
  54. Soussi, T., C. C. de Fromental, and P. May. 1990. Structural aspects of the p53 protein in relation to gene evolution. *Oncogene* 5:945-952.
  55. Stillman, B. 1989. Initiation of eukaryotic DNA replication *in vitro*. *Annu. Rev. Cell Biol.* 5:197-245.
  56. Takahashi, T., M. M. Nau, I. Chiba, M. J. Birrer, R. K. Rosenberg, M. Vinocour, M. Levitt, H. Pass, A. F. Gazdar, and J. D. Minna. 1989. p53: a frequent target for genetic abnormalities in lung cancer. *Science* 246:491-494.
  57. Thomas, R., L. Kaplan, N. Reich, D. P. Lane, and A. J. Levine. 1983. Characterization of human p53 antigen employing primate specific monoclonal antibodies. *Virology* 131:502-517.
  58. Vogelstein, B. 1990. Cancer: a deadly inheritance. *Nature (London)* 348:681-682.
  59. Wang, E. H., P. N. Friedman, and C. Prives. 1989. The murine p53 protein blocks replication of SV40 DNA *in vitro* by inhibiting the initiation functions of SV40 large T antigen. *Cell* 57:379-392.
  60. Werness, B. A., A. J. Levine, and P. M. Howley. 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 248:76-79.

# Wild-type mouse p53 down-regulates transcription from different virus enhancer/promoters

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The protein encoded by the tumour-suppressor gene p53 forms an complex with SV40 virus large T antigen, the adenovirus E1B 58-kDa protein and the E6 protein of human papillomavirus type 16. The functions of these complexes are unclear, but there is some evidence to suggest that binding of p53 to these viral proteins may inactivate p53 function. Recent reports have shown that p53 is involved in regulation of transcription. We have considered the possibility that p53 may regulate transcription of viral genes important for virus replication and/or transformation. Inactivation of p53 function by formation of such complexes might then permit correct expression of these viral genes. Since p53 can bind to the SV40 virus enhancer/promoter, we have investigated the effect of p53 on transcription from this promoter and report here that mouse p53 is a potent repressor of the SV40 enhancer/promoter. Mutations within p53 severely inhibited this activity and provided some evidence to show that the N-terminus of p53 contains residues essential for this function. We also show that mouse p53 represses transcription from the promoters of viruses that do not express proteins that complex with p53: the human cytomegalovirus early promoter and the Rous sarcoma virus long terminal repeat. By studying the effect of p53 on transcription in different cell lines, we show that the effects of p53 on promoters may be cell type specific.

## Introduction

There is now considerable evidence to suggest that the nuclear phosphoprotein p53, in its wild-type form (wt), acts as a tumour suppressor. For example, deletions and mutations of the p53 gene are associated with a wide variety of human tumours (Masuda *et al.*, 1987; Baker *et al.*, 1989; Nigro *et al.*, 1989; Sidransky *et al.*, 1991; Takahashi *et al.*, 1991). In addition, wt p53 is able to reduce the tumorigenicity of transformed cells (Chen *et al.*, 1991; Cheng *et al.*, 1992) and suppress the transformation of primary rat cells by several combinations of transforming genes, including mutant p53 and the *ras* oncogene (Finlay *et al.*, 1989). Consistent with this role as a tumour suppressor, wt p53 has been shown to inhibit proliferation of tumour cells (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990;

Michalovitz *et al.*, 1990; Casey *et al.*, 1991; Isaacs *et al.*, 1991). Interestingly, wt p53 is also able to inhibit replication of SV40 virus DNA *in vitro* and *in vivo* (Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988; Wang *et al.*, 1989).

The precise biochemical function of p53 is not yet clear. However, recent reports have provided evidence to suggest that at least one function of p53 may be in the regulation of transcription. Thus, studies in which the amino-terminal domain of p53 or intact p53 has been fused to the DNA-binding domain of the yeast GAL4 transcription factor have shown that p53 is able to stimulate transcription from a reporter gene containing multiple copies of the GAL4 DNA binding sites (Fields & Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990). This activity is dramatically reduced when wt p53 sequences are replaced by p53 mutants (Raycroft *et al.*, 1990; 1991; Unger *et al.*, 1992). Mouse p53 is also able to activate transcription from the mouse muscle-specific creatine kinase (MCK) gene promoter (Weintraub *et al.*, 1991). In contrast to these observations, a number of studies have clearly shown that wt p53 can down-regulate expression from the retinoblastoma gene promoter (Shiio *et al.*, 1992) and from the promoters of genes whose expression is controlled by growth factors, such as interleukin 6 (Santhanam *et al.*, 1991), *c-fos*, *c-jun* and  $\beta$ -actin (Ginsberg *et al.*, 1991). In addition, expression of wt p53 reduces levels of proliferating cell nuclear antigen (PCNA) mRNA and protein (Mercer *et al.*, 1991). Thus p53 appears to be both a positive and negative regulator of transcription.

One of the best-characterized features of p53 is its ability to interact with viral proteins. p53 has been shown to form complexes with the SV40 large T antigen (Lane & Crawford, 1979; Linzer & Levine, 1979), adenovirus type 5 E1B 58-kDa antigen in both transformed cells (Sarnow *et al.*, 1982; Zantema *et al.*, 1985) and infected cells (Braithwaite *et al.*, 1991a) and the E6 protein of human papillomavirus type 16 (HPV-16) (Werness *et al.*, 1990). The functional significance of these interactions is unclear, but there is some evidence to suggest that binding of the viral proteins to p53 may inactivate p53 function. For example, binding of p53 to the E6 protein of HPV-16 causes a rapid degradation of p53 (Scheffner *et al.*, 1990).

Since p53 appears normally to function as a transcriptional regulator, we have explored the possibility that one of the functions for the complexes formed between p53 and viral proteins may be to prevent p53 affecting key viral and cellular genes that are essential to viral replication and/or transformation. Consistent with this idea, the adenovirus E1B protein is able to

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prevent transcriptional activation by p53 (Yew & Berk, 1992).

Recent data have shown that p53 can bind to the SV40 virus enhancer/promoter (Bargonetti *et al.*, 1991). Therefore, we examined the effect of p53 on transcription driven from the SV40 enhancer/promoter using a transient expression system in which a reporter plasmid containing the bacterial chloramphenicol acetyl transferase (CAT) gene fused downstream of the SV40 enhancer/promoter (pSV2CAT) was co-transfected with vectors expressing mouse p53.

Under these conditions, we show that wt mouse p53 is a potent down-regulator of the SV40 enhancer/promoter and also of other viral enhancer/promoters. Mutations within p53 severely inhibited this down-regulation. In addition, using data from studies with our p53 mutants, we provide preliminary evidence that the N-terminal domain of p53 is essential for down-regulation. We also show that the effects of p53 on certain promoters may be cell type specific.

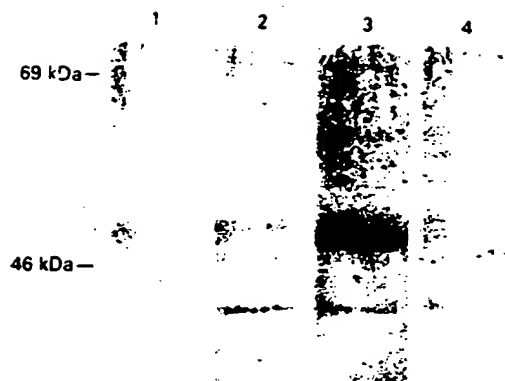
## Results

### *Specific down-regulation of transcription from the SV40 enhancer/promoter by mouse p53*

A number of reports have investigated the effect of p53 on transcription using a 'fusion protein' approach in which fragments of p53 are fused to the DNA-binding domain of GAL4 yeast transcription factor (see Introduction). In particular, Raycroft *et al.* (1990) showed that a GAL4-p53 fusion protein is able to activate transcription from a reporter plasmid containing multiple copies of the GAL4 DNA-binding sequence upstream of the SV40 promoter. We have been concerned that the data obtained from such 'fusion protein' approaches has not always reflected the true biological function of proteins (see for example Braithwaite *et al.*, 1991b). Therefore, to overcome any potential problems, we have investigated the effect of p53 on transcription from the SV40 enhancer/promoter without GAL4 binding sites in a transient transfection system. In this system, the reporter plasmid contained the SV40 enhancer/promoter inserted upstream of the bacterial CAT gene (pSV2CAT; Gorman *et al.*, 1982a). This plasmid was introduced into human HeLa cells, along with equal amounts of a plasmid expressing wt mouse p53 from the human cytomegalovirus (CMV) immediate-early promoter (pCMVNc9; Eliyahu *et al.*, 1989). HeLa cells were chosen because they do not express endogenous p53 protein (Benchimol *et al.*, 1982), which might complicate interpretation of results.

The absence of endogenous p53 protein in HeLa cells is probably due to the expression of E6 protein from human papillomavirus type 18 in these cells (Banks *et al.*, 1987), which is able to complex with and degrade human p53 protein (Scheffner *et al.*, 1990). It is possible that in our studies the introduced mouse p53 might also be degraded. Therefore, in an initial experiment, we confirmed expression of intact mouse p53 in HeLa cells after transfection, by labelling cells with [<sup>35</sup>S]methionine and immunoprecipitation of p53 from cells lysates using PAb 122 (Gurney *et al.*, 1980), a mouse p53 monoclonal antibody (Figure 1).

We next examined the effect of wt mouse p53 on the



**Figure 1** Expression of wt mouse p53 in transfected HeLa cells. HeLa cells were transfected with 20 µg of control plasmid (lanes 1 and 2) or 10 µg each of control plasmid and pCMVNc9 (lanes 3 and 4). After 72 h, cells were labelled for a further 2 h with [<sup>35</sup>S]methionine and cell lysates prepared. Lysates were then immunoprecipitated with either normal mouse serum (lanes 2 and 4) or PAb 122, a mouse-specific monoclonal antibody (lanes 1 and 3), as described in Materials and methods. Positions of molecular weight markers are indicated

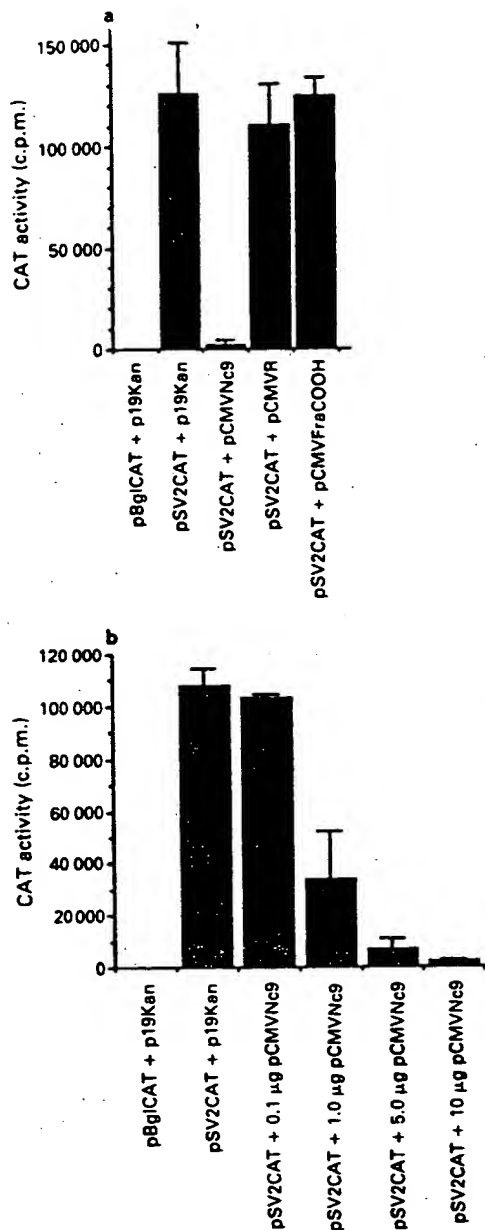
SV40 enhancer/promoter. Three days after co-transfection of pSV2CAT with either a control plasmid (p19Kan; Pridmore, 1987) or pCMVNc9, cell extracts were prepared and assayed for CAT activity by the method of Sleigh (1986). Results presented in Figure 2a indicate that CAT activity driven from the SV40 enhancer/promoter was more than 50-fold lower in cells co-transfected with pCMVNc9 than in cells transfected with the control. This effect of p53 was shown to be specific by replacing pCMVNc9 with either pCMVR (a plasmid containing the CMV promoter but encoding no protein) or pCMVFraCOOH (expressing residues 136–275 of the Fra-1 transcription factor, a protein unrelated to p53; Cohen *et al.*, 1989). There was no significant effect on CAT expression from the SV40 promoter/enhancer in cells co-transfected with either pCMVR or pCMVFraCOOH (Figure 2a).

By varying the amounts of pCMVNc9, we found a clear dose-dependent decline in SV40 enhancer/promoter activity with increasing amounts of plasmid expressing wt p53 (Figure 2b). A 50% inhibition of CAT activity was observed at approximately 0.75 µg of pCMVNc9 plasmid DNA.

In a separate set of experiments, we obtained similar, specific down-regulation of the SV40 enhancer/promoter in mouse L929 cells (fivefold reduction) and monkey CV1 cells (20-fold reduction), even though both cell lines express endogenous p53 (data not shown).

### *Kinetics of transcriptional down-regulation by p53*

Overexpression of wt p53 has been shown to arrest cellular growth at a point near the G<sub>1</sub>/S phase boundary of the cell cycle (Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Martinez *et al.*, 1991). Even though we were assaying CAT activity in lysates derived from equal numbers of cells, we were concerned that the effect of p53 on expression from the SV40 enhancer/promoter might be due to a secondary effect of slowed cell growth. Therefore, we performed a



**Figure 2** Specific down-regulation of the SV40 enhancer/promoter by mouse p53. (a) HeLa cells were co-transfected with 10  $\mu$ g each of the indicated plasmids, as described in Materials and methods. pBglCAT is a control plasmid that lacks the SV40 enhancer/promoter sequences upstream of the CAT gene (Gorman *et al.*, 1982a). p19Kan is a non-specific control plasmid (Pridmore, 1987). After 72 h, cell lysates were prepared, normalized for protein content (hence cell number) and used to determine CAT activity as described in Materials and methods. Data are presented as the means and standard deviations of three independent transfections, each assayed in duplicate. (b) HeLa cells were transfected with 10  $\mu$ g of CAT reporter plasmid and increasing amounts of pCMVNC9 as indicated. In all cases, the total amount of DNA transfected was maintained at 20  $\mu$ g by the addition of control plasmid. Data presented are the means with standard deviations of two independent transfections, each assayed in duplicate.

time-course experiment, in which cells were co-transfected with pSV2CAT and either a control plasmid or pCMVNC9. The results presented in Figure 3 show that in cells co-transfected with pSV2CAT and

the control plasmid no CAT activity was detected 8 h after transfection. However, low levels of CAT activity were detected by 24 h after transfection, which increased to a maximum after 72 h. In cells transfected with pSV2CAT and pCMVNC9, inhibition of the low levels of CAT activity observed 24 h after transfection (97%) was as prominent as the inhibition of the high levels of CAT activity observed 72 h after transfection. At this time (24 h), we found no difference in cell number between samples containing control plasmid or pCMVNC9 (data not shown). Although not conclusive evidence, these results suggest that the effect of p53 on the SV40 enhancer/promoter is not due to slowed cell growth.

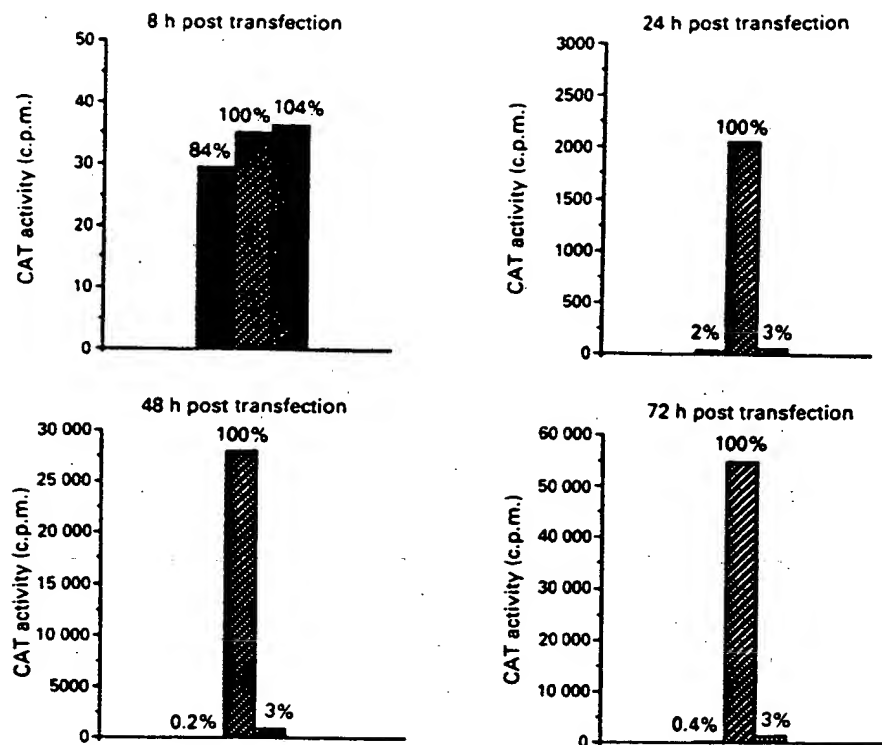
#### *p53 mutants have reduced ability to down-regulate transcription*

Recent reports have indicated that many p53 mutants have impaired ability to regulate either transcriptional activation (Raycroft *et al.*, 1990; 1991; Weintraub *et al.*, 1991; Kern *et al.*, 1992; Unger *et al.*, 1992) or repression (Santhanam *et al.*, 1991). We wanted to determine the effect of mutations within p53 on the ability of wt p53 to down-regulate transcription from the SV40 enhancer/promoter. Therefore, a number of plasmids that express mutant p53 proteins (Figure 4a) were co-transfected into HeLa cells along with pSV2CAT. The results presented in Figure 4b show that only pCMVNC9 and pCMVmsp53 were able to significantly reduce transcription from this promoter when compared with the control. The msp53 was, however, much less effective than wt p53, reducing activity only about fivefold. The ability of all other mutants, pCMVdl 163, pCMVdl 518 and pCMVc5, was abrogated.

To exclude the possibility that failure to down-regulate transcription was due to a failure of p53 expression in the transfected cells, HeLa cells were separately transfected with all the p53 constructs and labelled with [<sup>35</sup>S]methionine. Cell lysates were then immunoprecipitated with a monoclonal antibody specific for p53, PAb 122 (Gurney *et al.*, 1980). The results (Figure 4c) show that all constructs expressed a p53 protein of the expected size and that mutant proteins from pCMVdl 163, pCMVdl 518, and pCMVc5 were all expressed at levels equivalent to or greater than pCMVNC9 (lanes 2, 3, 4 and 6). Thus, loss of down-regulation of pSV2CAT by these mutant p53 proteins cannot be explained by failure to express p53. Mutant msp53, however, was expressed at a much lower level than pCMVNC9 (lanes 2 and 5), suggesting that the reduced ability of this mutant to down-regulate transcription may at least in part be due to the reduced levels of protein expression.

#### *wt p53 modulates the activity of other viral promoters*

p53 is able to complex with SV40 large T antigen, and we have shown in this report that mouse p53 is able to down-regulate expression from the SV40 enhancer/promoter. However, we were also interested to determine whether p53 is able to affect transcription from the promoters of viruses that do not express proteins that bind to p53. Therefore, reporter plasmids in which the bacterial CAT gene is fused upstream of the human



**Figure 3** Kinetics of transcriptional repression by p53. HeLa cells were co-transfected with 10  $\mu$ g of pBglCAT and 10  $\mu$ g of control plasmid, p19Kan (black bar), or 10  $\mu$ g of pSV2CAT with either 10  $\mu$ g of control plasmid (hatched bar) or pCMVNC9 (stippled bar). At the indicated times after addition of DNA, cells were harvested and assayed for CAT activity as described in Materials and methods

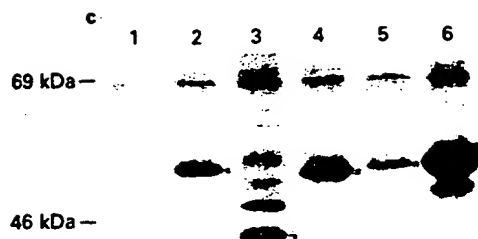
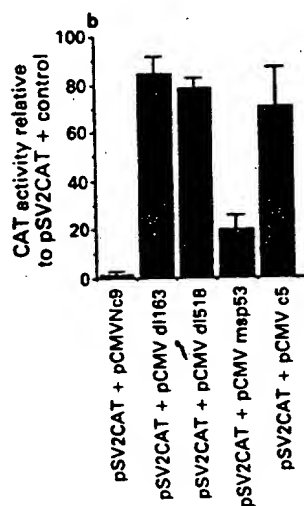
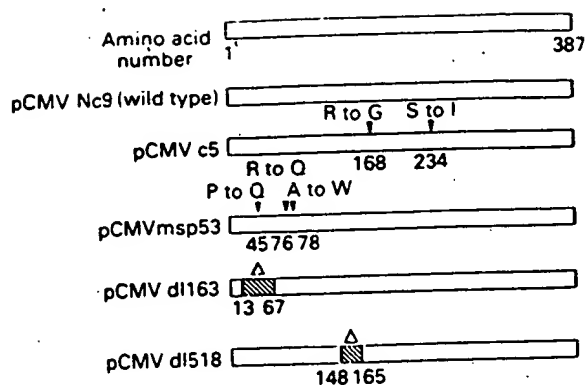
cytomegalovirus immediate-early promoter (pRcCMV-CAT) or the Rous sarcoma virus long terminal repeat (RSV-LTR) (pRSVCAT; Gorman *et al.*, 1982b) were co-transfected into HeLa cells with pCMVNC9. The data presented in Table 1 show that expression of p53 resulted in down-regulation of CAT expression from both the CMV promoter (twofold) and RSV-LTR (16-fold), though the levels of down-regulation were less than those observed for the SV40 enhancer/promoter. In addition, to ensure that the effects seen with p53 on the CMV and RSV promoters were not due to competition between promoters for limiting transcription factors, we replaced the control plasmid, p19Kan, with the plasmid pCMVR. We observed no reduction in activity obtained from these promoters (data not shown). These results demonstrate that down-regulation by p53 is not unique to the SV40 virus enhancer/promoter.

As a control for the effects of p53 expressed from pCMVNC9 on the viral promoters used in our system, we decided to analyse the effect of this p53 on the mouse muscle-specific creatine kinase (MCK) gene promoter. In monkey CV1 cells, this promoter has been shown to be activated by mouse p53 (Weintraub *et al.*, 1991). More specifically, by using increasing amounts of MCK promoter sequence, this group showed that p53 is able to activate the MCK promoter 10- to 80-fold when a 500-bp fragment containing the putative 'p53-responsive element' is present, but some activation (two- to fivefold) was also observed in the absence of this 500-bp sequence. The following MCK gene promoter-CAT constructs (Weintraub *et al.*, 1991) were

co-transfected into human HeLa cells, mouse L929 cells or monkey CV1 cells along with equal amounts of either a control plasmid or pCMVNC9: p3300MCK-CAT (containing promoter sequences from -3300 to +7), p2800MCKCAT (containing promoter sequences from -2800 to +7 and lacking the p53-responsive element) and p80MCKCAT (containing promoter sequences from -80 to +7). The results presented in Table 2 show that in CV1 cells we obtained levels of activation from the MCK promoter by mouse p53 in the absence (sixfold) and presence (20-fold) of the putative p53-responsive element that are similar to those observed by Weintraub *et al.* (1991). Interestingly, in HeLa cells and L cells, in which the levels of expressed p53 were much higher than that observed in CV1 cells (data not shown), we found that p53 caused no activation of the MCK promoter even when the p53-responsive element was present. Thus, some of the effects of p53 appear to be cell type specific. In addition, we consistently found that in all three cell types tested, but most clearly in CV1 and HeLa cells, p53 down-regulated the low levels of activity we obtained from the p80MCKCAT plasmid.

## Discussion

In this report, we have shown that wt mouse p53 is able specifically to down-regulate expression from the SV40 enhancer/promoter and the promoters of other viruses. p53 can also suppress transcription from the promoters of several growth factor-responsive genes



**Figure 4** Effect of deletions and point mutations in p53 on transcription from pSV2CAT. (a) Schematic illustration of p53 mutants used. Hatched areas indicate deletions. Abbreviations for amino acid residues are: A, Ala; G, Gly; I, Ile; P, Pro; Q, Gln; R, Arg; S, Ser; and W, Trp. p53 mutants dl 163, dl 518, msp53 and c5 were all expressed from the human CMV enhancer/promoter in plasmids pCMVdl163, pCMVdl518, pCMVmsp53 (Jenkins *et al.*, 1985; Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988) and pCMVc5 (Eliyahou *et al.*, 1989) respectively. (b) HeLa cells were co-transfected with the 10 µg of CAT reporter plasmid and 10 µg of either control plasmid or plasmid expressing p53, and assayed for CAT activity as described in Materials and methods. Results presented are the means with standard deviations of three independent transfections, each assayed in duplicate. (c) Expression of wild-type and mutant p53 proteins in transfected cells. HeLa cells were transfected with a control plasmid (lane 1) or plasmids expressing wt p53 (pCMVNc9) (lane 2), dl 163 (pCMVdl163) (lane 3), dl 518 (pCMVdl518) (lane 4), msp53 (pCMVmsp53) (lane 5) or c5 (pCMVc5) (lane 6). After 72 h, transfected cells were labelled with [<sup>35</sup>S]methionine for a further 2 h; cell lysates were then prepared and immunoprecipitated with PAb 122, a mouse p53 monoclonal antibody, as described Materials and methods. Asterisks indicate the precipitated p53 proteins. Positions of molecular weight markers are indicated

**Table 1** Regulation of transcriptional activity from different promoters by wt mouse p53

CAT reporter plasmid	Relative CAT activity*		
	Plasmids co-transfected with CAT reporter		
	p19Kan (control)	pCMVNc9 (wt p53)	Effect of p53
pSV2CAT	100	1.1 ± 0.04	91-fold reduction
pRSVCAT	100	6.1 ± 5.8	16-fold reduction
pRcCMVCAT	100	32.5 ± 17.6	2-fold reduction

\*HeLa cells were transfected with 10 µg of each of the indicated plasmids and assayed for CAT activity, as described in Materials and methods. In each case, levels of CAT activity obtained from cells transfected with pRSVCAT and pRcCMVCAT were similar to those obtained with pSV2CAT. Results presented are the means and standard deviations of three independent transfections, each assayed in duplicate

**Table 2** Modulation of transcriptional activity from the mouse MCK gene promoter by wt mouse p53

CAT reporter plasmid	Relative CAT activity*		
	Plasmids co-transfected with CAT reporter		
	p19Kan (control)	pCMVNc9 (wt p53)	Effect of p53
<b>CV1 cells</b>			
p80MCKCAT	1.00	0.2 ± 0.2	5-fold reduction
p2800MCKCAT	1.00	6.3 ± 1.6	6-fold activation
p3300MCKCAT	1.00	19.3 ± 0.8	19-fold activation
<b>HeLa cells</b>			
p80MCKCAT	1.00	0.1 ± 0.1	10-fold reduction
p2800MCKCAT	1.00	0.8 ± 0.6	No effect
p3300MCKCAT	1.00	1.6 ± 1.1	No effect
<b>L929 cells</b>			
p80MCKCAT	1.00	0.5 ± 0.3	No effect
p2800MCKCAT	1.00	1.4 ± 0.6	No effect
p3300MCKCAT	1.00	2.8 ± 2.2	3-fold activation

\*Cells were transfected with 10 µg of each of the indicated plasmids and assayed for CAT activity, as described in Materials and methods. Results presented are the means with standard deviations of two (CV1), three (L cells) and four (HeLa) independent transfections, each assayed in duplicate

(Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991), the retinoblastoma gene (Shiio *et al.*, 1992) and the multi-drug resistance gene (Chin *et al.*, 1992). The mechanism by which p53 down-regulates transcription is not clear. Recently, however, Shiio *et al.* (1992) identified a short, p53-responsive element in the retinoblastoma (Rb) gene promoter. This G(G/C)AA(G/C)TGA motif was required for specific down-regulation of the Rb promoter by p53. A similar sequence (GGAAGTGG) is present in the SV40 enhancer/promoter (+ 57 to + 64 of SV40 DNA) and is the centre of a domain to which both human and mouse p53 binds (Bargonetti *et al.*, 1991). Although we have no direct evidence, we would suggest that transcription from the SV40 enhancer/promoter is down-regulated by mouse p53 through binding to this octamer sequence.

Binding of p53 to a specific DNA sequence may be required for down-regulation of certain promoters, but there is also evidence to suggest that other mechanisms of down-regulation may be involved. Thus, p53 cannot bind DNA sequences from the promoters of growth factor-responsive genes (Santhanam *et al.*, 1991), and the p53-responsive element identified by Shiio *et al.*



(1992) is not present in the CMV and RSV-LTR promoters used in this study. It is also absent from both the published rat and human PCNA gene promoters (Travali *et al.*, 1989; Ohashi *et al.*, 1992) even though mouse p53 is able to down-regulate expression from the PCNA promoter (P. Jackson, unpublished data). In the absence of DNA binding, the mechanism of transcriptional repression is unclear. However, it is possible that p53 may act by interacting with one or more key transcription factors to prevent their binding to regulatory sequences.

p53 has been shown to activate transcription from both artificial promoters (containing elements of the SV40 promoter and the adenovirus E1B TATA box) (Fields & Jang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990) and natural promoters (Weintraub *et al.*, 1991). Recently, DNA footprinting has revealed that human p53 can bind two fragments of DNA containing a short, tandemly repeated sequence (Kern *et al.*, 1991). When placed upstream of an artificial promoter, the sequence enabled p53-specific activation of that promoter (Kern *et al.*, 1992). This p53-responsive element is also present in the mouse muscle-specific creatine kinase gene promoter (MCK) and allows p53 activation from this promoter (Weintraub *et al.*, 1991; this manuscript, Table 2). Interestingly, within each of the p53-binding fragments identified by Kern *et al.* (1991) are motifs similar to the consensus sequence identified by Shiiro *et al.* (1992): GGAAGTGT and GCAAGTC. The mechanism by which p53 can both activate and repress transcription from apparently similar sequences is not clear. Conceivably, p53 may act with similar factors for both activation and repression. Consistent with this notion, the amino-terminal domain of p53 appears essential for both transcriptional activation and repression. For activation, binding of p53 to the DNA through its specific element and to a particular factor(s) could promote binding of the transcription factor(s) to its regulatory element and/or alter the DNA conformation. In either case, the result is to activate a quiescent promoter. In contrast, in an active promoter, binding of p53 to its specific sequence may prevent binding of similar transcription factor(s), thereby inhibiting transcription.

In an initial series of experiments, Weintraub *et al.* (1991) showed that, in all cell types tested, wt mouse p53 was able to activate transcription from the mouse MCK promoter. Thus, p53 was able to activate the MCK promoter by similar amounts in monkey CV1 cells, human HepG2 cells and mouse CH3/10T $\frac{1}{2}$  cells. In contrast, in our experiments with CV1 cells, mouse L929 and human HeLa cells the effects of p53 show some cell type specificity, since p53 was unable to significantly activate transcription from the MCK promoter in either L cells or HeLa cells. These data would suggest that factors involved in transcriptional activation by p53 are also cell type specific, thus depending on the context p53 may or may not activate particular genes. The effects of p53 may also be species specific, since human wt p53 is unable to activate the MCK promoter in CV1 cells (Weintraub *et al.*, 1991) or down-regulate the SV40 enhancer/promoter in HeLa cells (Shiiro *et al.*, 1992). However, after having prepared this manuscript, Subler *et al.* (1992) reported that human wt p53 can down-regulate transcription from a number of viral and cellular promoters includ-

ing the SV40 enhancer/promoter, CMV enhancer/promoter and the RSV-LTR in NIH3T3 and VER cells. Again, these data would argue that cell-specific factors are important for p53 function.

A number of recent reports have shown that many p53 mutants have impaired ability to regulate either transcriptional activation (Raycroft *et al.*, 1990; 1991; Weintraub *et al.*, 1991; Kern *et al.*, 1992; Unger *et al.*, 1992) or repression (Santhanam *et al.*, 1991). Consistent with these observations, we have shown that mutations in most cases abrogate the ability of p53 to down-regulate transcription from the SV40 enhancer/promoter (Figure 4a and b). The loss of function observed for the mutants used in this study was not due to a failure to express the particular mutant protein (Figure 4c), though the reduced activity observed for pCMVmsp53 may well be explained by the fact that it was expressed at much lower levels than the wt p53.

The fact that the p53 mutants pCMVc5 and pCMVdl 518 have severely reduced ability to down-regulate the SV40 enhancer/promoter might suggest that the sequences within these p53 proteins affected by their mutations (both in the central region of the p53 protein) are important for the transcriptional down-regulation function of p53. However, it is also possible that these mutations alter the structure of the p53 protein and affect the structure of the true functional domain, which lies elsewhere on the protein. Consistent with this idea, these two mutant proteins fail to react with the conformation-sensitive monoclonal antibody PAb 246, whose epitope lies in the amino-terminal region of p53 (Jenkins & Sturzbecher, 1988). Rather than identifying a possible functional domain in p53, the simplest interpretation of the data for pCMVc5 and pCMVdl518 is that the mutants have adopted an overall unfavourable conformation for down-regulation. On the other hand, pCMVdl163 is in the wt conformation, as determined by its immunoreactivity with PAb 246 (Sturzbecher *et al.*, 1988), so the effect of the mutation in this p53 can be more clearly evaluated. pCMVdl163 almost fails to down-regulate the SV40 enhancer/promoter. These data suggest that residue 13-67 are important for the ability of mouse p53 to down-regulate the SV40 enhancer/promoter. Similar results for pCMVdl163 were obtained in experiments with the CMV enhancer/promoter and the RSV-LTR (P. Jackson, unpublished data). Although a more extensive series of mutants needs to be investigated, our preliminary conclusion is that an N-terminal domain of p53 may contain sequences responsible for down-regulation of the SV40 and other viral enhancer/promoters. Interestingly, this domain appears to be essential for transcriptional activation by p53 (Fields & Jang, 1990; Unger *et al.*, 1992) and is also the domain to which the adenovirus E1B 58-kDa protein binds (Braithwaite *et al.*, 1991a).

Recently, Yew & Berk (1992) reported that the adenovirus E1B 58-kDa protein can suppress p53-mediated activation of both an artificial promoter and the MCK promoter in BRK cells. This finding is consistent with the possibility that at least one of the functions for the interaction of viral proteins with p53 may be to overcome transcriptional regulation by p53. Hence, it will be of interest to identify whether or not p53 can affect transcription from any adenovirus gen-

promoter. Within the context of this report, it will also be of importance to determine whether or not E1B 5.5-kDa can overcome the p53-mediated down-regulation of the SV40 enhancer promoter.

## Materials and methods

### Cells

Human HeLa cells, mouse L929 cells and monkey CV1 cells were all routinely maintained in minimal essential medium (Gibco BRL, Grand Island, NY, USA) containing 0.22% sodium bicarbonate and 10% heat-inactivated fetal calf serum (FCS).

### Plasmids

Plasmids pSV2CAT expressing the bacterial CAT gene from the SV40 early enhancer/promoter (Gorman *et al.*, 1982a) and pBglCAT, a promoterless CAT plasmid (previously called pSV0, Gorman *et al.*, 1982a), were both generous gifts from M. Sleight (CSIRO, North Ryde, Australia).

Plasmid pCMVR, containing the human CMV immediate-early promoter but encoding no protein, and plasmid pCMVFra-COOH, encoding residues 136–275 of the Fra-1 protein expressed from the CMV immediate-early promoter (Cohen *et al.*, 1989), were gifts from D. Cohen (John Curtin School of Medical Research, Canberra, Australia).

Plasmids pCMVNC9 (Eliyahu *et al.*, 1989) encoding wild-type murine p53 and pCMVC5 (Eliyahu *et al.*, 1989) expressing murine p53 cDNA containing point mutations affecting amino acid residues 168 (R to G) and 234 (S to I) both expressed from the human CMV immediate-early promoter were obtained from M. Oren (Weizmann Institute, Rehovot, Israel).

Plasmid pRSVCAT expressing the CAT gene from the Rous sarcoma virus 3' LTR (Gorman *et al.*, 1982b) was obtained from B. van Leeuwen (John Curtin School of Medical Research, Australia).

The non-specific control plasmid p19Kan, a derivative of pUC19 in which the bacterial ampicillin resistance gene is replaced by a bacterial kanamycin resistance gene (Pridmore, 1987), was obtained from D. Pridmore, (Ciba-Geigy, Basle, Switzerland).

Plasmids encoding a mutant p53 lacking amino acid residues 148–165 (pCMVD1 518), lacking residues 13–67 (pCMVD1163) and with point mutations at residues 45 (R to Q), 76 (P to Q) and 78 (A to W) (pCMVMsp53JJ) (Jenkins *et al.*, 1985; Braithwaite *et al.*, 1987; Sturzbecher *et al.*, 1988), all expressed from the human CMV immediate-early promoter, were obtained from J. Jenkins (Marie Curie Research Institute, Oxford, UK).

Plasmid pRCMVCAT, expressing the bacterial CAT gene from the human CMV immediate-early promoter, was obtained from R. Reddel (Children's Medical Research Foundation, Sydney, Australia).

Plasmids containing sequences from the mouse-specific creatine kinase (MCK) gene promoter fused to the bacterial CAT gene, p3300MCKCAT, p2800MCKCAT and p80-MCKCAT (Weintraub *et al.*, 1991), were gifts from S. Hauschka (University of Washington, Seattle, WA, USA).

All plasmid DNA preparations for transfection were purified by caesium chloride equilibrium density centrifugation essentially as described in Sambrook *et al.* (1989).

### Transfection and assay of chloramphenicol acetyl transferase (CAT) activity

Cells were transfected with 10 µg of CAT reporter plasmid and 10 µg of either a control plasmid or p53 expression plasmid, essentially as described by Chen & Okayama (1987).

Briefly,  $5 \times 10^5$  cells were seeded into 10-cm Petri dishes and incubated overnight in 10 ml of growth medium. Plasmid DNA was then mixed with 0.5 ml of 0.25 M calcium chloride and 0.5 ml of  $2 \times$  BBS (50 mM BES pH 6.95, 280 mM sodium chloride, 1.5 mM disodium hydrogen phosphate) and the mixture incubated for 20 min at room temperature. The DNA solution was then added to the cells, dishes were gently swirled and incubated overnight in 3% carbon dioxide at 35°C. The medium was removed, cells were washed twice in growth medium, refed and incubation continued for a further 48 h at 5% carbon dioxide, 37°C, until harvested.

The level of CAT activity in transfected cell lysates was determined essentially as described by Sleight (1986). Each dish of transfected cells was washed twice in ice-cold phosphate-buffered saline (PBS). Cells were harvested by scraping into 1 ml of ice-cold PBS, pelleted and finally resuspended in 200 µl of 0.25 M Tris-Cl pH 7.8. Extracts of transfected cells were then prepared by three rounds of freezing (dry ice, 6 min) and thawing (37°C, 3 min). Cell suspensions were vortexed thoroughly before each freezing step. Cell debris was removed by centrifugation for 15 min at 12 000 g and 4°C. The supernatant was then heated to 65°C for 10 min to inactivate a CAT inhibitor previously identified by Sleight (1986).

To enable addition of lysates from equal numbers of cells from different samples into the CAT assay, the protein content in each cell extract was estimated by measurement of the absorbance at 280 nm.

CAT activity in cell lysates was assayed by measuring the transfer of [ $^{14}$ C]acetyl groups from [ $^{14}$ C]acetyl CoA into chloramphenicol as follows. Reaction mixtures contained cell lysate from transfected cells (normally approximately 30 µl), 20 µl of 8 mM chloramphenicol, 20 µl of diluted [ $^{14}$ C]acetyl CoA (60 mCi mmol $^{-1}$ , Amersham). Prior to use, 0.5 µCi (10 µl) of [ $^{14}$ C]acetyl CoA was diluted 10-fold with cold acetyl CoA (0.5 mM in 0.25 M Tris-Cl pH 7.5) and 0.25 M Tris-Cl pH 7.5 to a total volume of 100 µl. In each assay, volumes of cell lysate and 0.25 M Tris-Cl pH 7.5 were adjusted to account for differences in protein content so that the CAT activity was determined from equal numbers of cells from different samples. Reactions were allowed to proceed for 2 h at 37°C. Labelled products were then extracted into  $2 \times 10$  µl of ice-cold ethyl acetate. Layers were vigorously mixed and separated by centrifugation at 12 000 g for 3 min. After each extraction, the organic phase containing the labelled products was removed. To ensure no transfer of labelled substrate into the organic phase, a final back-extraction was performed on the combined organic phase with 100 µl of 0.25 M Tris-Cl pH 7.5. After vigorous mixing and centrifugation at 12 000 g, 100 µl of the organic phase was placed into 5-ml polyethylene vials, 4 ml of ReadySafe Scintillation Cocktail was added (Beckman Instruments, CA, USA) and the radioactivity determined by scintillation counting. In all experiments, radioactivity was measured as counts per min (c.p.m.) obtained from 5 min of counting.

### Immunoprecipitations

Petri dish cultures of transfected cells washed twice with PBS were incubated at 37°C, 5% carbon dioxide, in 2 ml of methionine-free minimal essential medium (Flow Laboratories, Irvine, UK) containing 1% L-glutamine and 2% FCS. After 30 min, the medium was removed, fresh methionine-free medium added and the incubation continued for a further 30 min. This medium was then removed, and the cells incubated in 2 ml of fresh methionine-free medium containing 100 µCi ml $^{-1}$   $^{35}$ S-Translabel (1192 Ci mmol $^{-1}$ , ICN Biomedicals, Irvine, CA, USA) for 2 h at 37°C, 5% carbon dioxide.

After removing the labelling medium and washing twice with ice-cold PBS, cells were lysed in 1 ml of RIPA buffer



(10 mM Tris-Cl pH 8.0, 150 mM sodium chloride, 1 mM EDTA, 1% NP-40, 0.1% SDS, 30  $\mu\text{g ml}^{-1}$  aprotinin). After 20 min. lysates were precleared at 120 000 g for 20 min. Immunoprecipitations were then carried out using protein A-Sepharose as described in Zhang *et al.* (1990). Precipitated proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

#### Antibodies

p53 proteins were immunoprecipitated from transfected cells with the monoclonal antibody Pab 122 (Gurney *et al.*, 1980). Normal mouse serum (NMS) was prepared from mice bred

at the John Curtin School of Medical Research under specific pathogen-free conditions.

#### Acknowledgements

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#### References

- Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., van Tuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., White, R. & Vogelstein, B. (1989). *Science*, **244**, 217–221.
- Baker, S.J., Markowitz, S., Fearon, E.R., Willson, J.K.V. & Vogelstein, B. (1990). *Science*, **249**, 912–915.
- Banks, L., Spence, P., Androphy, E., Hubbert, N., Matlashewski, G., Murray, A. & Crawford, L. (1987). *J. Gen. Virol.*, **68**, 1351–1359.
- Bargonetti, J., Friedman, P.N., Kern, S.E., Vogelstein, B. & Prives, C. (1991). *Cell*, **65**, 1083–1091.
- Benchimol, S., Pim, D. & Crawford, L. (1982). *EMBO J.*, **1**, 1055–1062.
- Braithwaite, A.W., Sturzbecher, H.-W., Addison, C., Palmer, C., Rudge, K. & Jenkins, J.J. (1987). *Nature*, **329**, 458–460.
- Braithwaite, A.W., Blair, G.E., Nelson, C.C., McGovern, J. & Bellett, A.J.D. (1991a). *Oncogene*, **6**, 781–787.
- Braithwaite, A.W., Nelson, C.C. & Bellett, A.J.D. (1991b). *New Biol.*, **3**, 18–26.
- Casey, G., Lo-Hsueh, M., Lopez, M.E., Vogelstein, B. & Stansbridge, E.J. (1991). *Oncogene*, **6**, 1791–1797.
- Chen, C. & Okayama, H. (1987). *Mol. Cell. Biol.*, **7**, 2745–2752.
- Chen, Y., Chen, P.-L., Arnaiz, N., Goodrich, D. & Lee, W.-H. (1991). *Oncogene*, **6**, 1799–1805.
- Cheng, J., Yee, J.-K., Yeargin, J., Friedmann, T. & Haas, M. (1992). *Cancer Res.*, **52**, 222–226.
- Chin, K.-V., Ueda, K., Pastan, I. & Gotteman, M. (1992). *Science*, **255**, 459–462.
- Cohen, D.R., Ferreira, P.C.P., Gentz, R., Franza Jr, B.R. & Curran, T. (1989). *Genes Dev.*, **3**, 173–184.
- Diller, L., Kassel, J., Nelson, C., Gryka, M., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S., Vogelstein, B. & Friend, S. (1990). *Mol. Cell. Biol.*, **10**, 5772–5781.
- Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O. & Oren, M. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 8763–8767.
- Fields, S. & Jang, S.K. (1990). *Science*, **249**, 1046–1049.
- Finlay, C.A., Hinds, P.W. & Levine, A. (1989). *Cell*, **57**, 1083–1093.
- Ginsberg, D., Mehta, F., Yaniv, M. & Oren, M. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 9979–9983.
- Gorman, C., Moffat, L. & Howard, B.M. (1982a). *Mol. Cell. Biol.*, **2**, 1044–1051.
- Gorman, C., Willingham, M.C., Pastan, I. & Howard, B.M. (1982b). *Proc. Natl. Acad. Sci. USA*, **79**, 6777–6781.
- Gurney, E.G., Harrison, R.O. & Fenno, J. (1980). *J. Virol.*, **34**, 752–763.
- Isaacs, W.B., Carter, B.S. & Ewing, C.M. (1991). *Cancer Res.*, **51**, 4716–4720.
- Jenkins, J.J., Rudge, K., Chumakov, P. & Currie, G.A. (1985). *Nature*, **317**, 816–818.
- Jenkins, J.J. & Sturzbecher, H.-W. (1988). In *The Oncogene Handbook*. Elsevier: Amsterdam, pp. 403–423.
- Kern, S.E., Kinzler, K.W., Bruskini, A., Jarosz, D., Friedman, P., Prives, C. & Vogelstein, B. (1991). *Science*, **252**, 1708–1711.
- Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, K., Kinzler, K.W. & Vogelstein, B. (1992). *Science*, **257**, 827–830.
- Lane, D.P. & Crawford, L.V. (1979). *Nature*, **278**, 261–263.
- Linzer, D.I.H. & Levine, A.J. (1979). *Cell*, **17**, 43–52.
- Martinez, J., Georgoff, I., Martinez, J. & Levine, A. (1990). *Genes Dev.*, **5**, 151–159.
- Masuda, H., Miller, C., Koeffler, H.P., Battifora, H. & Collins, M.J. (1987). *Proc. Natl. Acad. Sci. USA*, **84**, 7716–7720.
- Mercer, W.E., Shields, M., Amin, M., Suave, G.T., Appel, E., Ullrich, S. & Romano, J. (1990). *J. Cell Biochem.*, **41**, 285–290.
- Mercer, W.E., Shields, M.T., Lin, D., Appella, E. & Ullrich, S.J. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 1958–1962.
- Michalovitz, D., Halevy, O. & Oren, M. (1990). *Cell*, **61**, 671–680.
- Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.A., Hostetter, R., Cleary, K., Bigner, S.J., Davidson, I., Baylin, S., Devilee, P., Glover, T., Collins, F.S., West, A., Modali, R., Harris, C.C. & Vogelstein, B. (1986). *Nature*, **342**, 705–708.
- Ohashi, Y., Sawada, Y., Moriuchi, T. & Fujinaga, K. (1990). *Biochim. Biophys. Acta*, **1130**, 175–181.
- O'Rourke, R.W., Miller, C.W., Kato, G.J., Simon, K., Chen, D.-L., Dang, C.V. & Koeffler, H.P. (1990). *Oncogene*, **5**, 1829–1832.
- Pridmore, R.D. (1987). *Gene*, **56**, 309–312.
- Raycroft, L., Wu, H. & Lozano, G. (1990). *Science*, **249**, 1049–1051.
- Raycroft, L., Schmidt, J.R., Yoas, K., Hao, M. & Lozano, G. (1991). *Mol. Cell. Biol.*, **11**, 6067–6074.
- Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.
- Santhanam, U., Ray, A. & Sehgal, P.B. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 7605–7609.
- Sarnow, P., Ho, Y.S., Williams, J. & Levine, A.J. (1985). *Cell*, **28**, 387–394.
- Scheffner, S.M., Werness, B.A., Huibregtse, J.M., Levin, A.J. & Howley, P.M. (1990). *Cell*, **63**, 1129–1136.
- Shio, Y., Yamamoto, T. & Yamaguchi, N. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 5206–5210.
- Sidransky, D., Von Eschenbach, A., Tsai, Y.C., Jones, F., Summerhayes, I., Marshall, F., Paul, M., Green, F., Hamilton, S.R., Frost, P. & Vogelstein, B. (1991). *Science*, **252**, 706–709.
- Sleight, M.J. (1986). *Anal. Biochem.*, **156**, 251–256.
- Sturzbecher, H.-W., Brain, R., Maimets, Addison, C., Rudge, K. & Jenkins, J.J. (1988). *Oncogene*, **3**, 405–411.

- Subler, M.A., Martin, D.W. & Deb, S. (1992). *J. Virol.*, **66**, 4757-4762.
- Takahashi, T., Takahashi, T., Suzuki, H., Hida, T., Sekido, Y., Ariyoshi, Y. & Ueda, R. (1991). *Oncogene*, **6**, 1775-1778.
- Travali, S., Ku, D.-H., Rizzo, M.G., Ottavio, L., Baserga, R. & Calabretta, B. (1989). *J. Biol. Chem.*, **264**, 7466-7472.
- Unger, T., Nau, M.M., Segal, S. & Minna, J.D. (1992). *EMBO J.*, **11**, 1383-1390.
- Wang, E.H., Friedman, P.N. & Prives, C. (1989). *Cell*, **57**, 379-392.
- Weintraub, H., Hauschka, S. & Tapscott, S.J. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 4570-4571.
- Werness, B., Levine, A.J. & Howley, P.M. (1990). *Science*, **248**, 76-79.
- Yew, P.R. & Berk, A.J. (1992). *Nature*, **357**, 82-85.
- Zantema, A., Schrier, P.I., Davies-Olivier, A., Van Laar T., Vresen, R.T.M.J. & van der Eb, A.J. (1985). *Mol. Cell. Biol.*, **5**, 3084-3091.
- Zhang, X.L., Bellett, A.J.D., Tha Hla, R. & Braithwaite, A.W. (1990). *Virology*, **180**, 199-206.

# p53 represses SV40 transcription by preventing formation of transcription complexes

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There is now much evidence to suggest that the p53 tumour suppressor protein functions to monitor the integrity of the genome. When DNA damage is detected, p53 suppresses cell growth to allow repair or directs the cell into apoptosis. The mechanism of action of p53 is as yet unclear but recent evidence has accumulated to suggest that p53 might act by regulating gene expression. Consistent with this model, p53 can both activate and repress a number of viral and cellular promoters. p53 has also been shown to bind to the CCAAT-binding Factor and TATA-binding protein (TBP), and there is direct evidence that p53 represses *in vitro* transcription by preventing TBP from binding DNA. We now provide evidence that p53 can repress transcription from the SV40 promoter by disrupting DNA/protein complexes involving transcription factor Sp1.

**Keywords:** p53/SV40/gene repression

## Introduction

The p53 tumour suppressor gene encodes a nuclear phosphoprotein that appears to be functionally inactivated in an extraordinarily wide range of cancers (Hollstein *et al.*, 1991). The most frequent form of this inactivation is mutation which results in altered conformation of the p53 protein (Levine *et al.*, 1991). In fact, most mutations occurring in tumours result in the protein adopting a new and common 'mutant' conformation (Gannon *et al.*, 1990). An alternative form of inactivation occurs when p53 forms stable complexes with other proteins such as that which occurs in some cervical carcinomas (Vousden, 1993). In these cases, p53 remains wild type (wt) but is inactivated by binding to the E6 protein of oncogenic human papilloma viruses (Scheffner *et al.*, 1990; Vousden, 1993). The inactivation of p53 function is so widespread that it is now regarded as a hallmark of human malignancy.

The current and most powerful model of wt p53 function is one in which p53 monitors the genome for

DNA damage (Lane, 1992). If damage is detected, p53 slows cell growth (Baker *et al.*, 1990; Kuerbitz *et al.*, 1992) and activates DNA repair processes. In cancers, this monitoring process does not occur due to p53 inactivation. Thus, cells can continue to divide with damaged chromosomes. There is also evidence that in certain cell types p53 can cause apoptosis or programmed cell death in response to DNA damage caused by chemical agents and radiation (Clarke *et al.*, 1993; Lowe *et al.*, 1993).

There is now a considerable body of evidence that p53 can act as a transcription factor, which provides the basis for a potential mechanism by which p53 can inhibit cell growth (El-Deiry *et al.*, 1993) and possibly apoptosis (Shen and Shen, 1994). In this regard, p53 has been shown to both activate and repress a large number of viral and cellular promoters in reporter molecule assays (Ginsberg *et al.*, 1991; Santhanum *et al.*, 1991; Chin *et al.*, 1992; Kley *et al.*, 1992; Shiio *et al.*, 1992; Subler *et al.*, 1992; Agoff *et al.*, 1993; Jackson *et al.*, 1993, 1994; Ueba *et al.*, 1994; Miyashita *et al.*, 1994). Importantly, expression of the negative regulator of cell cycle progression, WAF1/CIP1 (El-Deiry *et al.*, 1993; Harper *et al.*, 1993), is activated by p53.

Although the molecular basis of transcriptional regulation by p53 is not yet fully understood, the pattern of results from many studies has indicated that activation of promoters requires p53 binding to specific DNA sequences. In contrast, repression of transcription appears to occur in the absence of p53 binding to DNA (see above references). Consistent with this idea, *in vitro* studies have demonstrated that wt p53 binds directly to the CCAAT-binding Factor (CBF; Agoff *et al.*, 1993) and TATA-binding protein (TBP; Seto *et al.*, 1992; Truant *et al.*, 1993) suggesting that p53 might repress transcription by interacting with these and possibly other transcription factors to prevent their binding to promoters. Indeed, this has been demonstrated directly in experiments where purified p53 was shown to prevent TBP from binding to its site in the *c-myc* promoter (Ragimov *et al.*, 1993).

In this paper, using the SV40 early enhancer/promoter as a model, we provide evidence that p53 prevents the formation of DNA/protein complexes involving transcription factors Sp1, AP-2 and TBP. Specifically, our data demonstrate that p53 can repress transcription by directly preventing the Sp1 transcription factor from binding to its target site within the SV40 promoter.

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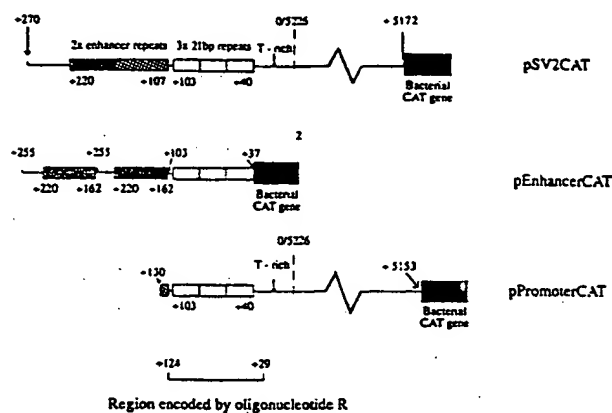
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## Results

### Location of a p53-responsive element within the SV40 promoter

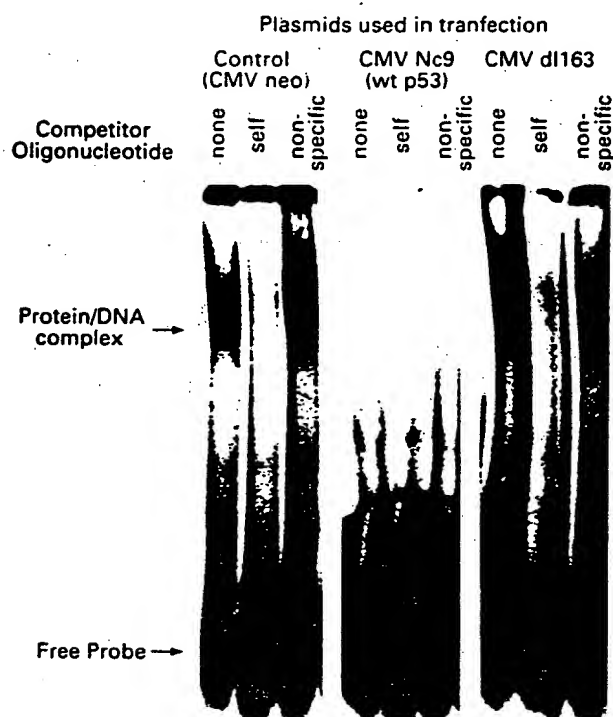
To identify a region within the SV40 promoter that is essential for p53 repression, we investigated the effect of p53 on the intact SV40 enhancer/promoter and on two promoter deletion constructs linked to the CAT reporter gene (Figure 1). Experiments were carried out by transfecting a number of different cell types with control and p53 expression plasmids along with appropriate reporter constructs. Qualitatively similar results were obtained in all cases. Thus the data obtained for HeLa cells only is shown in the following experiments.

As previously reported (Jackson *et al.*, 1993), activity from the intact SV40 enhancer/promoter was 20-fold lower in cells co-transfected with CMV Nc9 (expressing wt mouse p53) than in cells transfected with a control plasmid (CMV neo) or with a plasmid expressing a p53 mutant (CMV dl 163) (Table 1) which lacks part of the N-terminal transactivation domain. Expression of this mutant p53 construct has been examined many times in different cell types including HeLa, COS and rat embryo fibroblasts. In all cases the detectable p53



**Figure 1** Map of SV40 promoter/reporter constructs used in chloramphenicol acetyl transferase (CAT) assays. Numbers refer to the nucleotide position on the SV40 DNA early strand as previously defined (Tooze, 1980). CAT activity data obtained using these constructs is illustrated in Table 1

protein levels are at least comparable to those of a wt p53 expression construct. Examples of such data as determined by immunoprecipitation and immunoblotting have already been reported (Sturzbecher *et al.*, 1988b; Jackson *et al.*, 1993). Thus, the failure to repress transcription by dl 163 is not due to insufficient protein, but due to a specific defect in the protein. Furthermore, wt but not mutant p53, also repressed transcription from each of the SV40 promoter deletion constructs (Table 1). We conclude from these experiments that wt mouse p53 specifically represses the SV40 promoter and that since the minimum region of

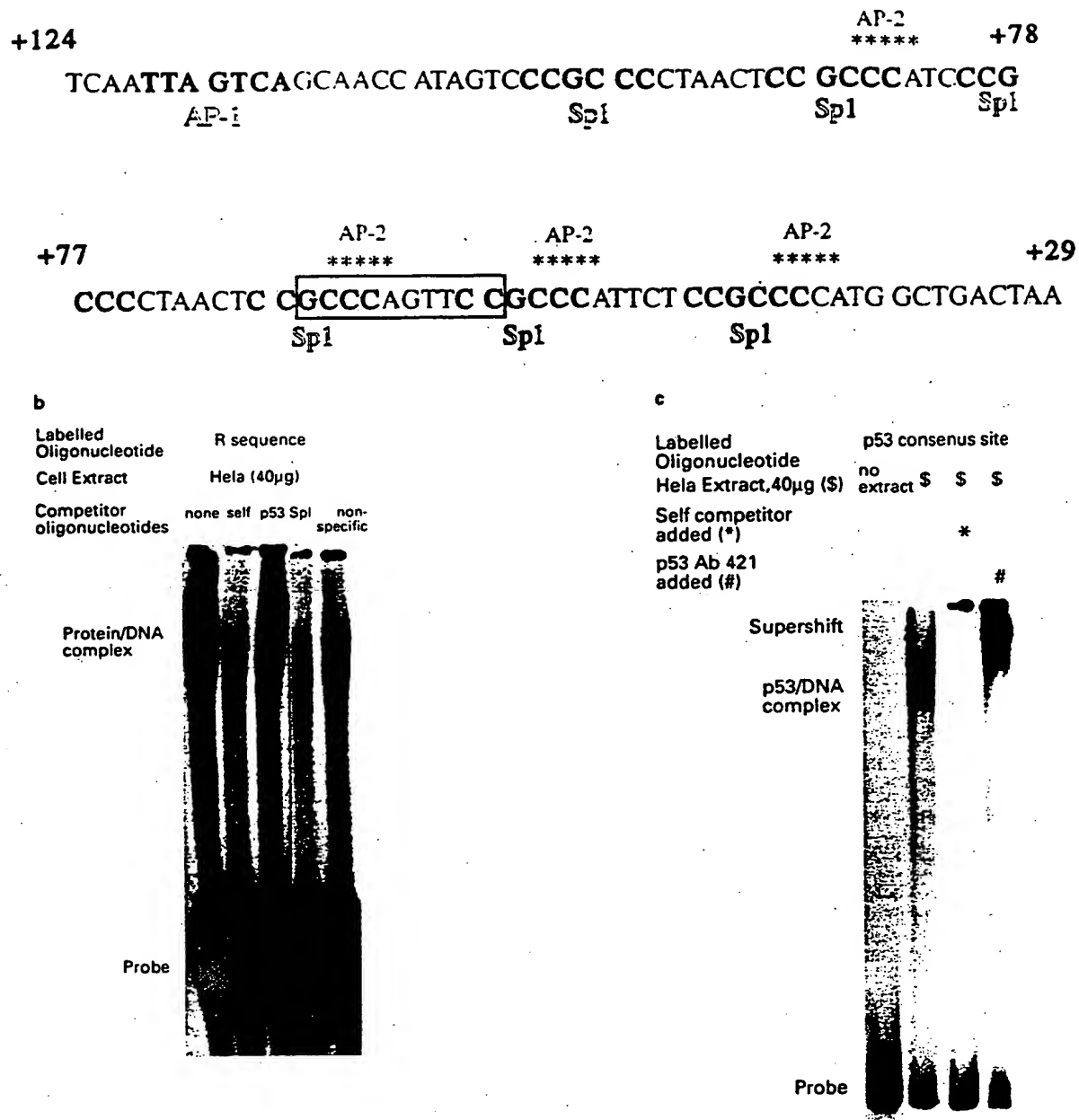


**Figure 2** Wild type but not mutant mouse p53 inhibits DNA/protein complex formation. Nuclear extracts from HeLa cells transfected with CMV neo, CMV Nc9 or CMV dl 163 were incubated with  $^{32}$ P-labelled oligo R (see below). This sequence contains a region sensitive to repression by wt mouse p53 (see Table 1). Binding reactions contained either no competitor, a 20-fold excess of unlabelled oligo R, or an unlabelled non-specific oligomer. Protein DNA complexes were resolved on a 5% polyacrylamide gel and the positions of the specific oligo R/protein complexes and unbound probe are indicated with arrows

**Table 1** Down-regulation of transcriptional activity from elements of the SV40 promoter by wt but not mutant mouse p53

Plasmids used in co-transfection	No of experiments	Relative activity ( $\pm$ s.d.)	-fold effect of p53
pSV2 CAT+CMV neo	7	100	
pSV2 CAT+CMV Nc9	7	$4.7 \pm 5.1$	20-fold repression
pSV2 CAT+CMV dl 163	1	140	no repression
pEnhancer CAT+CMV neo	8	100	
pEnhancer CAT+CMV Nc9	8	$14.2 \pm 11.4$	sevenfold repression
pEnhancer CAT+CMV dl 163	2	194	no repression
pPromoter CAT+CMV neo	6	100	
pPromoter CAT+CMV Nc9	6	$6.4 \pm 3.3$	17-fold repression
pPromoter CAT+CMV dl 163	2	109	no repression

Results of CAT analysis from cells transfected with SV40 promoter/reporter plasmids (see Figure 1) and either a control plasmid (CMV neo), CMV Nc9 (expressing wild type mouse p53) or CMV dl 163 (expressing a p53 deletion mutant lacking residues 14–66). Data represented are the mean with standard deviations of several experiments except for SV2CAT+CMV dl 163 which is the result of a single experiment since we have already reported that CMV dl 163 fails to down-regulate the SV40 enhancer/promoter (Jackson *et al.*, 1993).



**Figure 3** Multiple transcription factors bind to the R sequence of SV40 DNA but not p53. (a) The sequence of SV40 DNA from +29 to +124 (R sequence) with binding sites of transcription factors AP-1, AP-2 and Sp1 indicated. The boxed region indicates the putative p53 binding site reported by Bargonetti *et al.* (1991); (b) Sp1 but not endogenous HeLa cell p53 can bind to oligo R. Control HeLa cell extracts were incubated with <sup>32</sup>P-labelled oligo R with and without an excess of different competitor oligomers. There was a 50-fold excess of oligo R and Sp1 and a 200-fold excess of the p53 consensus oligomer (Kern *et al.*, 1991); (c) Endogenous HeLa p53 can bind a p53 consensus oligomer. Control HeLa cell extracts were incubated with a <sup>32</sup>P-labelled p53 oligomer in the absence or presence of a 50-fold excess of specific competitor, and in one instance in the presence of 500 ng of the p53 reactive antibody PAb 421 (Harlow *et al.*, 1981). Specific protein/DNA complexes were resolved on a 5% polyacrylamide gel and the positions of bound and free probe are indicated

overlap within the promoter deletion constructs is +37 to +130 (numbering from Tooze, 1980) (Figure 1), this region must contain a DNA sequence sufficient for p53-mediated repression.

#### *Mouse p53 inhibits protein factors binding to the p53-response element*

Since a number of transcription factors are predicted to bind to the region +37 to +130 within the SV40 promoter (Figure 3a), repression of the promoter might

be due to p53 interfering with the binding of such factors to this DNA sequence. Such a mechanism has been reported to explain the repression of the *c-myc* promoter by p53 (Ragimov *et al.*, 1993). This interference could occur by direct interaction with transcription factors or by competition for DNA binding sites. To investigate these possibilities, the sequence +29 to +124 (defined as sequence R; see Figure 3a) was amplified by the polymerase chain reaction (PCR) and then used in gel mobility shift assays. Nuclear extracts were prepared from HeLa cells

separately transfected with CMV neo, CMV Nc9 expressing wt mouse p53, or CMV dl 163 encoding the mutant p53 which failed to repress the SV40 promoter in the above reporter assays (Table 1).

Results (Figure 2) showed there to be a broadly migrating R sequence/protein complex present in extracts of cells transfected with CMV neo and CMV dl 163, suggesting that the complex is composed of multiple protein factors. However, this complex was barely detectable in extracts of cells transfected with CMV Nc9 (Figure 2). These data imply that wt mouse p53 in some way prevents the formation of protein/DNA complexes involving the R sequence. This result was reproducible using several different preparations of p53-containing nuclear extracts although the degree of complex inhibition varied between different preparations, presumably due to differences in transfection efficiency. Interestingly, we did not detect binding of p53 to the R sequence in this or other experiments, despite a report that both mouse and human recombinant p53 bind a site within the R sequence (Bargonetti *et al.*, 1991). This is explored in more detail below.

#### *Multiple transcription factors but not p53 bind to the p53-response element*

The sequence of the p53-responsive region from +37 to +130 included in the R sequence (Figure 3a) contains consensus AP-1 and Sp1 transcription factor binding sites (Dyran and Tjian, 1983; Chiu *et al.*, 1987), several low affinity AP-2 binding sites (Mitchell *et al.*, 1987) and a putative p53-binding site (Bargonetti *et al.*, 1991), but is upstream of a T-rich TATA-like sequence (Pauly *et al.*, 1992). Thus, given the data in Figure 2, a likely mechanism to account for the observed repression of the SV40 enhancer/promoter is that p53 binds to one or more of the above transcription factors and prevents binding to their target sites within the promoter. Since p53 can form self oligomers (Sturzbecher *et al.*, 1992), the transfected mouse p53 might be binding the endogenous p53 and disrupting or preventing assembly of the appropriate transcriptional initiation complex on the promoter. To determine if this was a possible mechanism, we took advantage of previous observations that HeLa cells express a low level of wt p53 protein, [unpublished data, Lehman *et al.* (1991) for sequence] which is capable of binding a p53 consensus oligomer (Hoppe-Seyler and Butz, 1993; Jackson *et al.*, 1995), to ask whether p53 can bind the R sequence under our conditions.

Competition gel shift experiments were performed using oligomers corresponding to consensus binding sites for p53 and Sp1 and using control HeLa cell extracts. Results (Figure 3b) showed that a p53 consensus oligomer, surprisingly, caused no reduction in R sequence/protein complex whereas self and Sp1 oligomers caused near complete loss of complex (Figure 3b). These data indicate that Sp1 is bound to the R sequence but that p53 is not. Other competition studies indicated that AP-1 and AP-2 are also present in the R sequence complex as expected from the sequence (data not shown).

Since these competition experiments indicated that p53 was not bound to the R sequence, a p53 consensus

oligomer was radiolabelled and used as a probe in a gel shift experiment to determine whether our conditions allowed DNA binding by p53. Results (Figure 3c) showed there to be clear p53 binding activity which is able to be 'super shifted' with the p53 specific monoclonal antibody PAb 421 (Harlow *et al.*, 1981). Similar results have been obtained with nuclear extracts of mouse cells (data not shown). Thus, the nuclear extracts we have used do contain p53 proteins capable of binding a consensus p53 site under our binding conditions, but which are not capable of binding the R sequence.

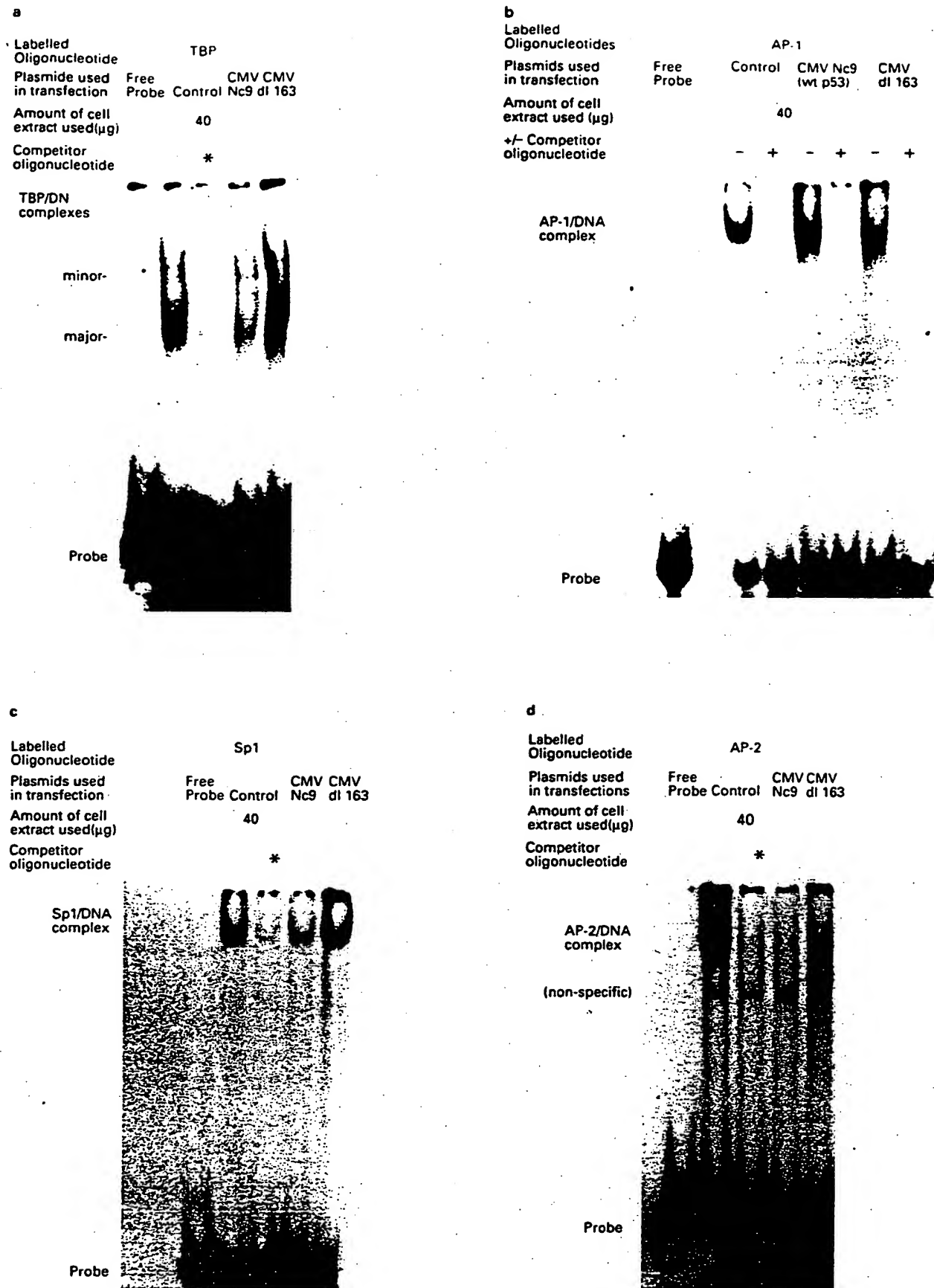
These data suggest that either (1) the R sequence is defective for p53 binding, (2) only recombinant p53 can bind within the R sequence, or (3) the binding of other transcription factors prevents p53 binding. The first possibility is unlikely to be correct because sequencing the amplified R fragment showed the sequence to be correct. It therefore seems likely that p53 fails to bind its putative site within the R sequence because other factors in some way interfere with its binding. This could occur by a combination of steric effects and by p53 being already bound to protein factors present in the extract. Presumably, the transfected mouse p53 also does not bind DNA (Figure 2) because it associates with protein factors in the extract, titrating out both its own DNA binding and that of other transcription factors. Significantly, the failure of p53 to bind to its site within the R sequence would be expected because p53 represses transcription from this promoter, whereas it is usual to obtain transactivation when p53 binds DNA (reviewed in Donehower and Bradley, 1993).

#### *p53 prevents TBP, Sp1, AP-2 but not AP-1 from binding DNA*

To investigate which of the above transcription factors other than endogenous p53 might be the target for wt mouse p53, consensus oligomers corresponding to the binding sites for each of the above transcription factors were used in gel mobility shift assays. Again, these experiments were carried out using extracts of HeLa cells transfected with CMV neo (control), CMV Nc9 and CMV dl 163.

The first set of experiments was carried out with the consensus oligomer for TBP because p53 has been reported to prevent TBP binding its DNA motif (Ragimov *et al.*, 1993). Results in Figure 4a showed a major and a minor protein/DNA complex present in extracts of cells transfected with control and mutant p53 plasmids. Both complexes were substantially reduced in extracts containing wt mouse p53. This result therefore confirms the previous report that mouse p53 can prevent formation of a TBP/DNA complex.

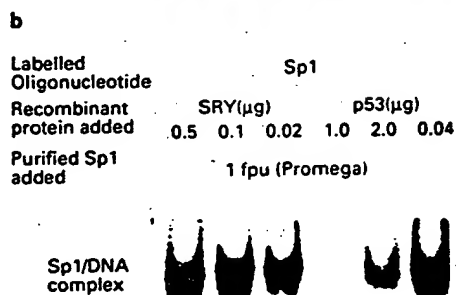
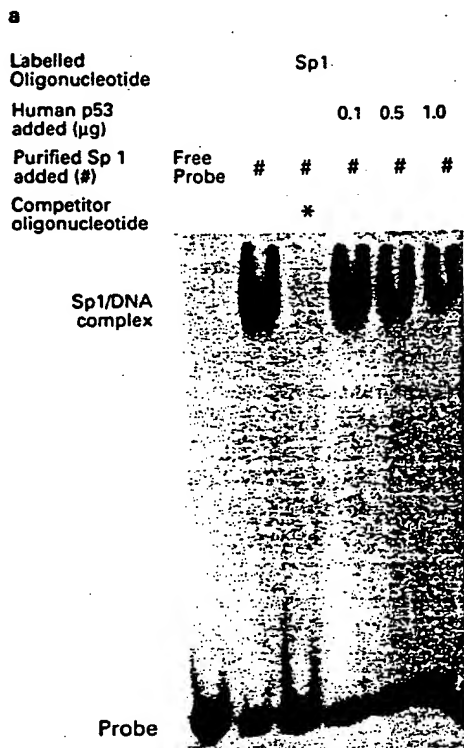
When a similar experiment was carried-out with a consensus oligomer for transcription factor AP-1 (Figure 4b), a single retarded DNA-protein complex was evident in all three extracts. Thus, unlike the results with TBP, the data with the AP-1 consensus oligomer suggest that mouse p53 cannot prevent formation of AP-1/DNA complexes. This result has been confirmed in mixing studies using *in vitro* translated p53 and AP-1 proteins (unpublished observations).



**Figure 4** Wild type p53 inhibits protein/DNA complex formation involving AP-2, Sp1 and TBP. Nuclear extracts from transfected cells were mixed with  $^{32}$ P-labelled oligomers corresponding to the binding sites for transcription factors TBP (a), AP-1, (b), Sp1 (c) and AP-2 (d). Binding reactions contained no specific competitor or a 36-fold (AP-1), 25-fold (Sp1), 36-fold (TBP) or 100-fold (AP-2) excess of the same unlabelled oligomer (tracks indicated by an asterisk). Specific protein/DNA complexes were resolved on a 5% polyacrylamide gel. Although not shown here, for all studies, irrelevant control oligonucleotide competitors have been used to validate the specificity of the binding reactions

Consensus oligomers for the binding sites for Sp1 were also used in gel mobility shift assays with transfected cell extracts. For Sp1, wt mouse p53 containing extracts showed considerably reduced binding compared to control or mutant p53 transfected extracts (Figure 4c), similar to the findings with the TBP binding site.

However, in a similar experiment with the AP-2 consensus site, whilst wt p53 completely abolished binding compared with control extracts, mutant dl 163 containing extracts also caused a substantial inhibition of binding (Figure 4d). This result suggests that p53 interacts with AP-2 in a different way from TBP and Sp1.



These data suggest that wt p53 can inhibit the binding of Sp1, AP-2 and TBP but not AP-1 to their respective DNA binding sites. In addition, mutant p53 dl 163, also appears to inhibit AP-2 binding but is unable to inhibit the binding of any of the other transcription factors.

#### p53 directly interferes with Sp1 but not AP-2 binding

The results of experiments shown above suggest that p53 directly interferes with the binding of certain transcription factors to the SV40 promoter thereby causing repression of transcription. However, the data do not exclude the possibility that p53 induces expression of some intermediate protein which is responsible for interference with transcription factor binding. As the minimum response sequence (R sequence, Figure 3a) does not contain a TBP binding site and p53 is unable to inhibit AP-1 binding, only studies of Sp1 and AP-2 binding were expanded upon. To distinguish between the possibilities that p53 may interact directly with these transcription factors, or may act through another factor, gel mobility shift experiments were set up using purified recombinant human Sp1, AP-2 and p53 proteins. In this instance, human wt p53 was used instead of mouse p53 which was used in the transfection experiments. Importantly, however, CAT assays have shown that human p53 expressing constructs also repress transcription from

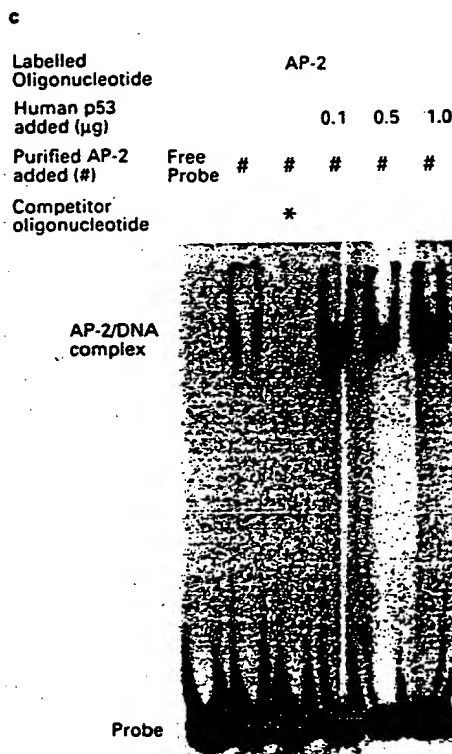


Figure 5 Recombinant p53 prevents Sp1 but not AP-2 binding DNA. Binding of purified Sp1 (1 foot printing unit (fpu), Promega) to its DNA consensus sequence was measured in the presence of increasing amounts of recombinant human p53 (a,b) or the sex-determining factor SRY (b). Binding of purified AP-2 (1 fpu, Promega) was also measured in the presence of increasing amounts of recombinant human p53 (c). Specific competitor was again present in tracks marked by an asterisk. Purified proteins were incubated in the presence of 0.05 µg of the synthetic polymer poly(dI-dC).poly(dI-dC)



the SV40 promoter although less potently (data not shown).

Consistent with our findings using transfected cell extracts, the results of gel shift experiments showed that recombinant wt human p53 interfered with the binding of purified Sp1 to its binding site in a dose dependent manner (Figure 5a and b). However, over the same molar concentration range, the HMG box binding protein SRY (sex-determining factor), which has transcriptional regulatory properties (Cohen *et al.*, 1994), did not prevent Sp1 from binding DNA (Figure 5b). These data strongly suggest that p53 and not some intermediate, is directly and specifically responsible for preventing Sp1 from binding DNA. Although quantitatively more recombinant p53 is used in these experiments than is present in the nuclear extracts used in Figure 4, there is sufficient p53 in the extracts to prevent the same amount of recombinant Sp1 binding DNA (data not shown) as was used in Figure 5. The reason for the quantitative difference is most likely because recombinant p53 is not as potent as cellular p53.

When similar experiments were carried-out with recombinant AP-2, its binding was not prevented by addition of recombinant p53 protein to the binding reaction (Figure 5c). This suggests that p53 is probably not directly responsible for interfering with AP-2 binding.

## Discussion

Experiments reported in this paper have investigated a possible mechanism for transcriptional repression mediated by p53 using the SV40 enhancer/promoter as a simple and well-defined model. With the use of promoter/reporter constructs (Figure 1) we identified a region of 93 bp. from +37 to +130, which contains a DNA element sufficient for p53-mediated repression (Table 1). A PCR generated DNA fragment encompassing most of this region (R sequence, Figure 3a) was then used in gel mobility shift assays using nuclear extracts from HeLa cells transfected with CMV neo, CMV Nc9 expressing wt mouse p53 and CMV dl 163 expressing a mutant p53 which fails to repress the SV40 reporter constructs (Table 1). Control and mutant p53 containing extracts showed a broadly migrating R sequence/protein complex (suggesting the presence of multiple transcription factors) which was essentially absent from extracts containing wt mouse p53 (Figure 2).

These two sets of data, combined with the observations that the R sequence contains binding sites for several transcription factors (Figure 3a) suggested that p53 represses the SV40 promoter by interfering with transcription factors binding to promoter elements within this sequence. Competition gel shift experiments using oligomers corresponding to the consensus binding sites for all the predicted transcription factors, showed that Sp1, AP-1 and AP-2 were bound to the R sequence, but p53 was not (Figure 3b), despite the fact that the extracts did contain p53 proteins competent to bind a p53 consensus site under our conditions (Figure 3c). Thus, it seemed likely that the other transcription factors were in some way preventing the binding of p53

to its site in the R sequence by steric interference and/or by direct binding of p53. These other transcription factors thus seemed likely targets for the introduced mouse p53 and again probably account for the failure of the transfected p53 to bind the R sequence.

The possibility that mouse p53 targets one of these other factors was tested using gel shift experiments with transfected cell extracts and radiolabelled oligomers corresponding to the binding sites for TBP, Sp1, AP-1 and AP-2. The results from these experiments showed that wt p53 containing extracts inhibited binding of all these transcription factors with the exception of AP-1 (Figure 4a-d).

These data therefore suggest that repression of the SV40 enhancer/promoter is due to interference by wt p53 in transcription complex formation involving the DNA sequence +37 to +130 and at least one of the transcription factors AP-2 or Sp1 (there is no TBP site within our defined region of +37 to +130). Sp1 would seem to be the more likely candidate as its DNA binding is prevented by wt p53 containing extracts (Figure 4c), but not by extracts containing the mutant p53 (Figure 4c) which fails to repress the SV40 promoter (Figure 1). In addition, p53 appears to directly interfere with Sp1 binding as indicated from studies with purified proteins (Figure 5a and b). AP-2 could also be a candidate as it too is prevented from binding to its site by wt p53 (Figure 4d). However, the fact that the mutant which does not repress the SV40 promoter, does prevent AP-2 binding, suggests that loss of AP-2 binding does not compromise promoter activity. Thus, given the above considerations, the simplest explanation of our data is that p53 represses transcription from the SV40 promoter by binding Sp1 and preventing it from binding DNA. Consistent with this interpretation, Sp1 binding has been shown to be critical for activity of the SV40 promoter (Dyran and Tjian, 1983) and also has been found to coimmunoprecipitate with p53 (Borellini and Glazer, 1993) indicating the two proteins can form a complex.

The observation that the R sequence complex is completely absent from wt p53 containing extracts (Figure 2) even though only Sp1 appears to be targeted by p53 is paradoxical. A possible explanation for this, is that Sp1 is the 'keystone' of the complex so that if this factor is lost, the complex is completely disrupted. This explanation is consistent with the competition experiments in which an Sp1 oligomer caused near complete loss of R sequence complex (Figure 3b).

Examination of the promoter sequences of the IL6 (Ray *et al.*, 1988),  $\beta$ -actin (Quitschke *et al.*, 1989), HIV LTR (Nabel *et al.*, 1988) and HSP 70 (Wu *et al.*, 1986) gene promoters has also identified a combination of AP-2, Sp1 and TBP binding sites. Interestingly, all of these promoters are repressed by p53. p53 can also repress a separate class of promoters which are TATA-less, for example the p53 promoter itself (Bienz-Tadmor *et al.*, 1985; Ginsberg *et al.*, 1991), and which possess GC-rich sequences containing potential Sp1 and AP-2 binding sites. Our data therefore suggest that interference in binding of AP-2, Sp1 and/or TBP is a common mechanism for repression of basal promoter activity by p53. Such interactions may well be sufficient to explain how p53 is able to repress such a diverse collection of both cellular and viral gene promoters.

## Materials and methods

### Cell culture

HeLa cells (clone ATCC CCL2) were routinely maintained in Minimal Essential Medium (MEM; Gibco BRL, Grand Island, New York, USA) containing 0.22% sodium bicarbonate and 10% foetal calf serum in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

### Plasmids

Plasmids SV<sub>2</sub>CAT (Gorman *et al.*, 1982), CMV neo (Braithwaite *et al.*, 1987) containing the Tn5 transposon conferring resistance to the neomycin/kanamycin family of antibiotics (Southern and Berg, 1982), pEnhancer CAT and pPromoter CAT (pCAT-enhancer and pCAT-promoter; Promega), CMV Nc9 expressing wild type mouse p53 (Eliyahu *et al.*, 1989) and CMV dl 163 expressing a mouse p53 mutant deleted between amino acids 13 and 67 (Jenkins *et al.*, 1985; Braithwaite *et al.*, 1987; Sturbecher *et al.*, 1988a) have already been described. CMV dl 163 expresses a stable protein product in transfected HeLa (Jackson *et al.*, 1993) and COS cells (Sturbecher *et al.*, 1988b) to at least wt levels.

### Transfections

For chloramphenicol acetyl transferase (CAT) assays, about 5 × 10<sup>5</sup> HeLa cells were seeded into 90 mm dishes and subsequently transfected using a modified calcium phosphate method (Chen and Okayama, 1987) with 10 µg of reporter and 10 µg of p53-expression plasmid. For gel mobility shift assays, about 3 × 10<sup>6</sup> cells were seeded into 75 cm<sup>2</sup> flasks and transfected using the same procedure but with 30 µg of expression plasmid and 25 µg of carrier DNA. 72 h after transfections, cells were extracted and assayed for CAT activity or used in gel shift assays. Details of the transfection procedure have been described previously (Jackson *et al.*, 1993).

### Polymerase chain reaction (PCR)

Oligonucleotide R containing the putative p53-response element was amplified by PCR using pPromoterCAT as a template with a 25 bp 5' primer, 5'GGAGCTTTCAAT-TAGTCAGCAACCA-3', a 26 bp 3' primer, 3'-GCGGGGTACCGACTGATTGACGTCGG-5' and Taq polymerase (Pharmacia) used exactly as described by the manufacturer.

### CAT assays

CAT activity was measured as previously described (Sleigh, 1986) and the details of our procedures have been reported previously (Jackson *et al.*, 1993). All assays were normalized for protein content.

### Gel mobility shift experiments

HeLa cells transfected as described with CMV neo, CMV Nc9 or CMV dl 163, as well as nontransfected controls, were incubated for 72 h at 37°C and nuclear extracts

prepared (Schreiber *et al.*, 1989). Once prepared, extracts were dialyzed overnight against 500 volumes of 20 mM Tris-HCl pH 7.0, 10% glycerol, 1 mM EDTA, 40 mM NaCl, 1 mM DTT (TEG). Gel mobility shift assays were performed by incubating 40 µg of nuclear extract with 10 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 12.5% glycerol, 0.1% Triton-X-100 (Borellini and Glazer, 1993) and 1 µg of the synthetic polynucleotide poly(dI-dC).poly(dI-dC) for 15 min at 20°C. Unless otherwise indicated, during this time any competitors were also included. Labelled oligonucleotide probe (10 000 c.p.m.) was added and the reaction continued for a further 15 min. Protein/DNA complexes were then resolved on 5% polyacrylamide gels which were fixed in 10% acetic acid, dried under vacuum at 80°C for 25 min and exposed to Kodak XAR-5 film at -70°C.

When binding reactions were carried-out using purified recombinant human AP-2, Sp1 (both purchased from Promega) and p53 (see below), conditions were as described above but without cell extract.

### Purification of p53 protein

Human p53 was purified from *E. coli* containing pET19b-hup53. A single colony was inoculated into LBamp medium overnight and induced in 400 ml of LBamp with 1.0 mM IPTG at O.D.<sub>600</sub> of 0.5. The cells were incubated at 37°C for 3 h and harvested by centrifugation at 8000 g for 10 min at 4°C. The cells were then lysed for 1 h at 4°C in 6 M guanidinium/HCl, 50 mM Tris-HCl, pH 8.0. The lysate was cleared by centrifugation at 12 000 g for 20 min at 4°C and the supernatant filtered through gauze. The supernatant was then incubated overnight with 750 ml of Ni<sup>2+</sup> Nitrilotriacetic acid (NTA)-agarose equilibrated with the guanidinium lysis buffer. The agarose beads were pelleted at 1000 g and washed five times with the lysis buffer. The beads were then dialysed against p53 buffer (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM β-2-Mercaptoethanol, 0.1% Triton-X-100, 7.5% glycerol and 300 mM NaCl) containing 1 M guanidinium, subsequently 0.1 M guanidinium and then without guanidinium. Upon transfer to a column and washing with five volumes of p53 buffer (pH 6.3), 500 µl fractions were eluted with p53 buffer pH 5.0 and collected in eppendorfs containing 17 µl of 500 mM Tris-HCl, pH 8.0. Column fractions were then analysed by SDS-PAGE and the purified protein was subsequently dialysed against 500 volumes of TEG. Aliquots were stored at -70°C.

### Acknowledgements

We thank Donna Cohen for a generous gift of recombinant SRY and David Tremethick for reagents and much useful advice. Roseanne Hansen is also warmly thanked for her intelligent and detailed comments on the manuscript. This work was supported in part by a grant from the Australian Tobacco Research foundation to AB and PJ.

### References

- Agoff SN, Hou J, Linzer DIH and Wu B. (1993). *Science*, 259, 84-87.
- Baker SJ, Markowitz S, Fearon ER, Willson JKV and Vogelstein B. (1990). *Science*, 249, 912-915.
- Bargonetti J, Friedman PN, Kern SE, Vogelstein B and Privet C. (1991). *Cell*, 65, 1083-1091.
- Bienz-Tadmor B, Zakut-Houri R, Libresco S, Givol D and Oren M. (1985). *EMBO J.*, 4, 3209-3213.
- Borellini F and Glazer RI. (1993). *J. Biol. Chem.*, 268, 7923-7928.
- Braithwaite AW, Sturbecher H-W, Addison C, Palmer C, Rudge K and Jenkins JR. (1987). *Nature*, 329, 458-460.

- Chen C and Okayama H. (1987). *Mol. Cell. Biol.*, 7, 2745-2752.
- Chin K-V, Ueda K, Pastan I and Gottesman M. (1992). *Science*, 255, 459-462.
- Chiu R, Imagawa M, Imbra RJ, Backoven JR and Karin M. (1987). *Nature*, 329, 648-651.
- Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH. (1993). *Nature*, 362, 849-852.
- Cohen DR, Sinclair AH and McGovern JD. (1994). *Proc. Natl. Acad. Sci. USA*, 91, 4372-4376.
- Donchower LA and Bradley A. (1993). *Biochim. Biophys. Acta*, 1155, 181-203.
- Dynan WS and Tjian R. (1983). *Cell*, 35, 79-97.
- El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell*, 75, 817-825.
- Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O and Oren M. (1989). *Proc. Natl. Acad. Sci. USA*, 86, 8763-8767.
- Gannon JV, Greaves RG, Iggo R and Lane DP. (1990). *EMBO J.*, 9, 1595-1602.
- Ginsberg D, Mechta F, Yaniv M and Oren M. (1991). *Proc. Natl. Acad. Sci. USA*, 88, 9979-9983.
- Gorman C, Moffat and Howard BM. (1982). *Mol. Cell. Biol.*, 2, 1044-1051.
- Harlow E, Crawford LV, Pim DC and Williamson NM. (1981). *J. Virol.*, 39, 861-869.
- Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ. (1993). *Cell*, 75, 805-816.
- Hollstein M, Sidransky D, Vogelstein B and Harris CC. (1991). *Science*, 253, 49-53.
- Hoppe-Seyler F and Butz K. (1993). *J. Virol.*, 67, 3111-3117.
- Jackson P, Bos E and Braithwaite AW. (1993). *Oncogene*, 8, 589-597.
- Jackson P, Ridgway P, Rayner J, Noble J and Braithwaite A. (1994). *Biochem. Biophys. Res. Commun.*, 203, 133-140.
- Jackson P, Shield M, Buskin J, Hawkes S, Reed M, Perrem K, Hauschka SD and Braithwaite A. (1995). *Gene Expression*, 5, in press.
- Jenkins JR, Rudge K, Chumakov P and Currie GA. (1985). *Nature*, 317, 816-818.
- Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C and Vogelstein B. (1991). *Science*, 252, 1708-1711.
- Kley N, Chung RY, Fay S, Loeffler JP and Seizinger BR. (1992). *Nucl. Acids Res.*, 20, 4083-4087.
- Kuerbitz SJ, Plunkett BS, Walsh WV and Kastan MB. (1992). *Proc. Natl. Acad. Sci. USA*, 89, 7491-7495.
- Lane DP. (1992). *Nature*, 358, 15-16.
- Lehman TA, Bennett WP, Metcalf RA, Welsh JA, Ecker J, Modali RV, Ullrich S, Romano JW, Appella E and Testa JR. (1991). *Cancer Res.*, 51, 4090-4096.
- Levine AJ, Momand J and Finlay CA. (1991). *Nature*, 351, 453-456.
- Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T. (1993). *Nature*, 362, 847-849.
- Mitchell PJ, Wang C and Tjian R. (1987). *Cell*, 50, 847-861.
- Miyashita T, Masayoshi M, Hanada M and Reed JC. (1994). *Cancer Res.*, 54, 3131-3135.
- Nabel GJ, Rice SA, Knipe DM and Baltimore D. (1988). *Science*, 234, 1299-1302.
- Pauly M, Treger M, Westhof E and Chambon P. (1992). *Nucl. Acids Res.*, 20, 975-982.
- Quitschke WW, Lin Z-Y, Deponti-Zilli L and Paterson BM. (1989). *J. Biol. Chem.*, 264, 9539-9546.
- Ragimov N, Krauskopf A, Navot N, Rotter V, Oren M and Aloni Y. (1993). *Oncogene*, 8, 1183-1193.
- Ray A, Tatter SB, May LT and Sehgal PB. (1988). *Proc. Natl. Acad. Sci. USA*, 85, 6701-6705.
- Santhanum U, Ray A and Sehgal PB. (1991). *Proc. Natl. Acad. Sci. USA*, 88, 7605-7609.
- Scheffner SM, Werness BA, Huibregste JM, Levine AJ and Howley PM. (1990). *Cell*, 63, 1129-1136.
- Schreiber E, Matthias P, Muller MM and Schaffner W. (1989). *Nucl. Acids Res.*, 17, 6419.
- Seto E, Usheva A, Zambetti GP, Momand J, Horikoshi N, Weinmann R, Levine AJ and Shenk T. (1992). *Proc. Natl. Acad. Sci. USA*, 89, 12028-12032.
- Shiio Y, Yamamoto T and Yamaguchi N. (1992). *Proc. Natl. Acad. Sci. USA*, 89, 5206-5210.
- Shen Y and Shenk T. (1994). *Proc. Natl. Acad. Sci. USA*, 91, 8940-8944.
- Sleigh MJ. (1986). *Anal. Biochem.*, 156, 251-256.
- Southern PJ and Berg PJ. (1982). *Molec. Appl. Genet.*, 1, 327-341.
- Sturzbecher H-W, Brain R, Maimets T, Addison C, Rudge K and Jenkins JR. (1988a). *Oncogene*, 3, 405-413.
- Sturzbecher H-W, Braithwaite AW, Addison C, Palmer C, Rudge K, Lynge-Hansen D and Jenkins JR. (1988b). *Cancer Cells*, 6, 159-163.
- Sturzbecher H-W, Brain R, Addison C, Rudge K, Remm M, Grimaldi M, Keenan E and Jenkins JR. (1992). *Oncogene*, 7, 1513-1523.
- Subler MA, Martin DW and Deb S. (1992). *J. Virol.*, 66, 4757-4762.
- Tooze J. (1980). *DNA Tumor Viruses*, Pt.2, Tooze J. (ed.), Cold Spring Harbor Laboratory, New York.
- Truant R, Xiao H, Ingles CJ and Greenblatt J. (1993). *J. Biol. Chem.*, 268, 2284-2287.
- Ueda T, Nosaka T, Takahashi JA, Shibata F, Florkiewicz RZ, Vogelstein B, Oda Y, Kikuchi H and Hatanaka M. (1994). *Proc. Natl. Acad. Sci. USA*, 91, 9009-9013.
- Vousden KH. (1993). *FASEB J.*, 7, 872-879.
- Wu BJ, Kingston RE and Morimoto RI. (1986). *Proc. Natl. Acad. Sci. USA*, 83, 629-633.

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**Differential effects of butyrate derivatives on human breast cancer cells grown as organotypic nodules in vitro and as xenografts in vivo.****Planchon P, Magnien V, Beaupain R, Mainguene C, Ronco G, Villa P, Brouty-Boye D**

Institut d'Oncologie Cellulaire et Moléculaire Humaine, Hopital Avicenne, Bobigny, France.

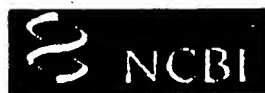
The antiproliferative and cytodifferentiating effects of a new stable butyric derivative, monobut-3, were compared using human MDA-MB-231 breast cancer cells grown in three dimension as either in vitro tumor nodules or in vivo xenograft tumors. In in vitro tumor nodules, monobut-3 exhibited marked growth inhibitory effects consistent with the results obtained in monolayer cell cultures. Some functional cell differentiation was also detected in treated nodules. In in vivo xenografts, monobut-3 significantly decreased MDA-MB-231 tumor take but did not affect the rate of tumor growth. No difference was noted in the histological characteristics of the xenografts between untreated and treated mice. Moreover, once monobut-3 treatment was discontinued, tumor growth rapidly resumed in tumor-free animals. The decreased efficacy of monobut-3 in in vivo MDA-MB-231 xenografts as compared to in vitro tumor nodules indicates that factors related to host environment may still limit the clinical effectiveness of this compound.

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**Pharmacodynamics of cisplatin in human head and neck cancer: correlation between platinum content, DNA adduct levels and drug sensitivity in vitro and in vivo.**

Related Resources

Welters MJ, Fichtinger-Schepman AM, Baan RA, Jacobs-Bergmans AJ, Kegel A, van der Vijgh WJ, Braakhuis BJ

Toxicology Division, TNO Nutrition and Food Research Institute, Zeist, The Netherlands.

Total platinum contents and cisplatin-DNA adduct levels were determined in vivo in xenografted tumour tissues in mice and in vitro in cultured tumour cells of head and neck squamous cell carcinoma (HNSCC), and correlated with sensitivity to cisplatin. In vivo, a panel of five HNSCC tumour lines growing as xenografts in nude mice was used. In vitro, the panel consisted of five HNSCC cell lines, of which four had an in vivo equivalent. Sensitivity to cisplatin varied three- to sevenfold among cell lines and tumours respectively. However, the ranking of the sensitivities of the tumour lines (in vivo), also after reinjection of the cultured tumour cells, did not coincide with that of the corresponding cell lines, which showed that cell culture systems are not representative for the in vivo situation. Both in vitro and in vivo, however, significant correlations were found between total platinum levels, measured by atomic absorption spectrophotometry (AAS), and tumour response to cisplatin therapy at all time points tested. The levels of the two major cisplatin-DNA adduct types were determined by a recently developed and improved 32P post-labelling assay at various time points after cisplatin treatment. Evidence is presented that the platinum-AG adduct, in which platinum is bound to guanine and an adjacent adenine, may be the cytotoxic lesion because a significant correlation was found between the platinum-AG levels and the sensitivities in our panel of HNSCC, in vitro as well as in vivo. This correlation with the platinum-AG levels was established at 1 h (in vitro) and 3 h (in vivo) after the start of the cisplatin treatment, which emphasizes the importance of early sampling.

PMID: 10408697, UI: 99335182

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**Immunoliposome-mediated targeting of doxorubicin to human ovarian carcinoma in vitro and in vivo.**

Vingerhoeds MH, Steerenberg PA, Hendriks JJ, Dekker LC, Van Hoesel QG, Crommelin DJ, Storm G

Related Resources

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands.

This paper deals with the utility of immunoliposomes for the delivery of doxorubicin (DXR) to human ovarian carcinoma cells in vitro and in vivo. We aimed to investigate whether immunoliposome-mediated targeting of DXR to ovarian cancer cells translates in an enhanced anti-tumour effect compared with that of non-targeted DXR liposomes (lacking the specific antibody). Target cell binding and anti-tumour activity of DXR immunoliposomes were studied in vitro and in vivo (xenograft model of ovarian carcinoma). In vitro we observed that target cell binding and cell growth inhibition of DXR immunoliposomes is superior to that of non-targeted DXR-liposomes. However, in vivo, despite the efficient target cell binding and good anti-tumour response of DXR-immunoliposomes, no difference in anti-tumour effect, compared with non-targeted DXR-liposomes, could be determined. The results indicate that premature DXR leakage from immunoliposomes occurring before the actual target cell binding and subsequent DXR association with the tumour cells, explains why no significant differences in anti-tumour activity between DXR-immunoliposomes and non-targeted DXR-liposomes were observed in vivo.

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## The effect of high dose vitamin A on the morphology and proliferative activity of xenograft lung and head and neck cancer.

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Mourad WA, Bruner JM, Vallieres E, McName C, Alabdulwahed S, Scott K, Oldring DJ

Department of Pathology, University of Alberta, Edmonton, Canada.

In vitro studies have suggested that vitamin A lowers invasive potential of squamous cell carcinoma. Epidemiological data have also indicated that high dose vitamin A may improve survival in patients with previously resected lung carcinoma. To our knowledge, no studies have attempted to test the in vivo effect of vitamin A on the morphology and growth rate of lung and head and neck cancer. Freshly resected tumor cell suspensions were obtained by ex vivo fine needle aspiration and injected subcutaneously in duplicate in athymic male nude mice. Two to six weeks post-engraftment tests and controls were separated for each xenograft. Mice with test xenografts were given water soluble vitamin A (Aquasol ATM, Astra pharmaceutical, Westborough, MA, U.S.A) at a dose of 10,000 U/Kg/day intraperitoneally for 6 to 10 weeks (median 8 weeks). One to two hours prior to sacrifice bromodeoxyuridine (BrdU) was injected intraperitoneally to assess the S-phase fraction in both test and control xenografts. Blood vitamin A levels in test and control animals were measured after sacrifice using high performance liquid chromatography (HPLC). Sections of test and control xenografts were routinely stained to assess morphologic differentiation and mitotic counts. Unstained sections of xenografts were immunostained by the antibody to BrdU to test for BrdU labeling index (BLI) reflecting S-phase fraction (SPF) and also by the MIB-1 antibody to assess proliferative activity. Eighteen tumors were studied. These included 9 squamous cell carcinomas of the lung, 5 squamous cell carcinomas of the head and neck, and 4 adenocarcinomas of the lung. Blood levels of vitamin A in test animals were 7 to 23 times those of the control animals (median 13 times). Neovascularization of the xenografts was seen in all cases. The morphology and mitotic activity of the test and control xenografts showed no significant difference. SPF and proliferative activity measured by BrdU and MIB-1 immunolabelling respectively showed no significant difference between test and control xenografts. Our study suggests that there is no significant in vivo effect of high dose vitamin A on the morphology and growth rate of xenografted non small cell carcinoma of the lung or squamous cell carcinoma of the head and neck.



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## Transforming growth factor-beta and response to anticancer therapies in human liver and gastric tumors in vitro and in vivo.

**Liu P, Menon K, Alvarez E, Lu K, Teicher BA**

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**Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA**

Liver cancer and gastric cancer are the most common solid tumors worldwide. Transforming growth factor-beta (TGF-beta) production and lack of response to TGF-beta growth inhibitory effects have been associated with tumor progression and therapeutic resistance. HepG2, Hep3B, and SK-HEP-1 human liver cancer lines produce 3, 5.7, and 2.5 ng TGF-beta1; 1.4, 2, and 4 ng TGF-beta2 and 0.15, 0.2 and 0.22 ng TGF-beta3 per 107 cells (24 h). Expression of the TGF-beta type I receptor is 20x, 1x, and 0.6x the level in mink lung MvLu1 cells in the HepG2, Hep3B, and SK-HEP-1 cells, respectively. HepG2 and Hep3B cells do not express the TGF-beta type II receptor while SK-HEP-1 cells express 7x the level found in mink lung MvLu1 cells. Hs 746T, KATO III, RF-1, and RF-48 human gastric cancer cell lines produce 12, 5, 0.35, 0.4, and 0.4 ng TGF-beta1; 2.6, 0.95, 0.5, and 0.52 ng TGF-beta2 and 0.42, 0.17, 0.12, and 0.14 ng TGF-beta3 per 107 cells (24 h). Expression of TGF-beta type I receptor is 0.7x, 0.7x, 0.8x, 0.6x the level in mink lung MvLu1 cells in the Hs 746T, KATO III, RF-1 and RF-48 cells, respectively. KATO III cells are lacking in the TGF-beta type II receptor while Hs 746T, RF-1 and RF-48 cells express 10x, 0.8x, and 1x the levels in mink lung MvLu1 cells. The IC50 for TGF-beta1 is >>10 ng/ml in all of these lines except RF-48 where TGF-beta1 is mitogenic. The response of the cell lines to radiation, doxorubicin, mitomycin C, cisplatin, 5-fluorouracil, methotrexate, and gemcitabine showed that SK-HEP-1 was the most drug resistant liver cancer cell line and KATO III was the most drug resistant gastric cancer cell line. Overall, there was no correlation between TGF-beta secretion in cell culture and sensitivity of the cells to anticancer agents. Increased TGF-beta1 levels were detectable in the plasma of nude mice bearing Hep3B and Hs 746T xenografts. Those tumors which secreted greater amounts of TGF-beta were more therapeutically resistant in vivo.

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**Tumour-growth suppression in nude mice by a murine monoclonal antibody: factors hampering successful therapy.**

Johansson C, Segren S, Lindholm L

Pharmacia CanAg, Goteborg, Sweden.

The murine MAb C215 has been shown to mediate ADMMC in vitro and to have a tumour-growth-suppressive effect on xenografted COLO 205 human colocal carcinoma cells in nude mice. To overcome the limitations of MAb therapy, it is necessary to understand the underlying mechanisms of tumour-growth suppression. In the present work, we have used C215 to define the importance of different parameters involved in tumour therapy with murine IgG2a antibodies. The results show that there exists a period of roughly 2 days after inoculation into animals during which the tumour cells are sensitive to an inhibitory antibody-mediated effect. After this initial period, the in-vivo sensitivity of tumour cells to antibody-mediated inhibition is much reduced. Tumour cells can remain "dormant" and, despite ongoing antibody treatment, develop into tumours with a reduced growth rate, which is not caused by outgrowth of antigen-deficient tumour cells. Finally, a pronounced dependence of antibody-mediated tumour suppression on antibody dose was observed.

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